## *The molecular basis of storage organs in Brassicas*

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# The molecular basis of storage organ development in Brassicas

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## i. Abstract

The *LEAFY COTYLEDON* (*LEC*) family of genes are intrinsically linked to the formation of the *Arabidopsis* embryo in morphogenesis and maturation. They play several roles in this process including maintenance of embryonic identity and the formation of storage products in the seed. This is important for desiccation protection and as an energy store for germination and growth before photomorphogenesis occurs. Loss of function mutations have been shown to exhibit non-embryonic traits such as trichomes and anthocyanin sequestration. When *LECs* are expressed outside of embryonic tissues in Arabidopsis, accumulation of storage oils and immature cell identity is observed. They appear to exhibit large amounts of crosstalk with the hormones auxin and gibberellins.

This thesis investigates the link between *Leafy Cotyledon 1* (*LEC1*) expression in the *Arabidopsis* turnip (*tnp*), a gain-of-function mutant of *LEC1*, and hypocotyl development in selected *Brassica sp.*

In particular;

Are *LECs* ectopically expressed in the hypocotyl of the Brassicas post germination?

To what extent are *LEC1*, *LEC2* and *FUSCA3* (*FUS3*) expressed in relation to one another?

Is the timing of starch and lipid accumulation linked temporally to *LEC* expression?

Fatty acid profiles of the *tnp* mutant were explored in relation to the wild type and further investigations and comparisons to profiles in the Brassica hypocotyl tissues were examined.

Relatively high ectopic *LEC* expression was observed in the Brassica hypocotyl tissue. *LEC1* was found to be associated with hypocotyl expansion and starch accumulation. *LEC2* expression levels increase after *LEC1* levels decrease. The same oils observed to increase in the *tnp* mutant of Arabidopsis are present in large amounts in the Brassica hypocotyl tissue, suggesting a potential link between *LECs* and the molecular basis of storage organ development in Brassicas.

## ii. Declaration and Statement of copyright



## iii. Acknowledgements

I would like to thank my supervisors Keith and Jen for their support throughout the project, even when I had a tendency to injure myself and drag the experiments on for far too long.

I would also like to thank the rest of the members of the lab group: Anna, James, Flora and Vinny for their patience and technical support, even when they had experiments to run and theses of their own to write. Thank you to Kat and Amy who worked on their Masters' alongside me, all of whom could be somewhat scathing and insulting about the proper etiquette of formal attire, but made the experience much more enjoyable.

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# 1 Introduction

## 1.1 Embryogenesis Overview

The sporophytic generation of higher plants begins with the formation of a single celled zygote and the progenitor cell in the endosperm following a double fertilisation event (West & Harada, 1993). Embryogenesis is defined as the period of development after fertilisation resulting in the formation of the mature embryo. This is the result of several morphological and cellular changes; resulting in formation of cotyledons (the number dependent on whether the plant is di or monocot) and an embryonic axis with shoot and root polls (West & Harada, 1993). Normally, this will also contain high levels of storage molecules such as starches and fats. This prepares the seed for long periods of dormancy and acts as a nutrient source after germination.

In order for embryogenesis to take place, a number of developmental processes must occur. These include morphogenesis: the elaboration of basic forms, organogenesis: the formation of functionally organised structures, and histogenesis: the differentiation of cells contained within various tissues (Taiz & Zeiger, 2006).

Within the past century significant work has been undertaken on higher plant embryos. Light and electron microscopy has given characterised work on the anatomical changes throughout development (Maheshwari, 1950; Natesh & Rau, 1984). Work on cellular differentiation has been mainly focused on biosynthesis and the accumulation of storage molecules such as starch and glycolipids for use after germination and skotomorphogenesis (Casey et al., 1986; West & Harada, 1993). A large amount of work has now also been done on early embryonic morphogenesis and patterning (Lindsey & Topping, 1993).

Patterns within embryogenesis differ between monocotyledons and dicotyledons. The members of the *Brassica* family to be investigated are all dicotyledonous and the stages of development can be seen in Figure 1.

The development process is split into two stages after the initial double fertilisation. The first stage is morphogenesis, F – K Figure 1, where the body plan of the plant is established as well as the development of cellular differentiation and radial symmetry (Casson & Lindsey, 2006). The second is maturation where morphogenesis is arrested in seed plants, causing the accumulation and sequestration of storage molecules in order both to protect the embryo from desiccation during dormancy and to provide nutrients after germination (Braybook and Harada, 2008).

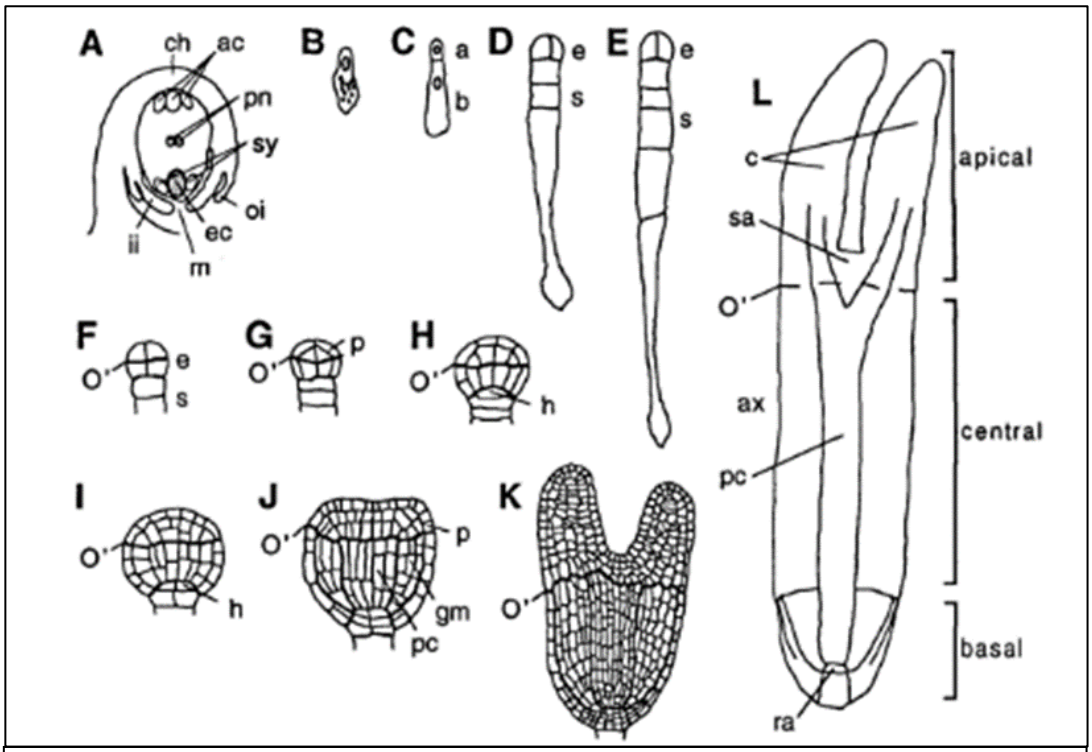



**Figure 1.** Embryonic Development in a Representative Dicotyledonous Plant. (A) Ovule. (B) Zygote. (C) One-celled embryo proper. (D) Two celled embryo proper. (E) Quadrant stage embryo. The two-celled embryo proper divides by another longitudinal division, perpendicular to the plane of the previous division in the embryo, to produce a four-celled embryo proper. The suspensor has undergone additional transverse divisions. (F) Octant stage embryo. The four quadrants have divided by transverse divisions to produce an eight-celled embryo proper. The transverse cell walls produced in this division form the indicated O' line. The basal portion of the suspensor is not shown. (G) Dermatogen stage. The cells of the octant stage embryo have divided by cleavages parallel to the surface to form a sixteen-celled embryo proper, setting apart the protoderm (p). (H) Early globular stage. The cells of the protoderm have undergone division's perpendicular to the surface. The interior cells of the embryo proper have undergone additional longitudinal divisions. The topmost cell of the suspensor has divided transversely to produce the hypophysis (h). (I) Mid-globular stage. The cells of the hypophysis have divided longitudinally. The cells in the interior of the embryo proper have divided both longitudinally and transversely, while the protodermal cell divisions have continued. (J) Transition stage. Cell divisions parallel to the surface indicate the emergence of the cotyledon buttresses as the apical pole of the embryo becomes broader. The developing procambium (pc) becomes visible as elongated cells at the centre of the embryo. gm, ground meristem. (K) Heart stage. Cotyledonary lobes continue to enlarge, making the change to bilateral symmetry more obvious. The O' line is still recognizable. (L) Linear cotyledon stage. The morphological organization of the embryo is shown. The apical domain comprises the cotyledons (c), the shoot apex (sa), and the upper axis; the central domain consists of the bulk of the axis (ax); and the basal domain includes the root apex (ra). The developing vascular tissue forks just below the O' boundary.
Figure taken from West & Harada, (1993).

## 1.2 The Role of *LECs* in Embryogenesis

### 1.2.1 *LECs* in maturation

The LEAFY COTYLEDON (LEC) transcription factors (TFs) establish conditions that allow the promotion of cellular processes that are characteristic of the maturation phase and the initiation of somatic embryo formation (Braybook and Harada, 2008).

LEC1, LEC2, FUSCA3 (FUS3) and ABSCISIC ACID INSENSITIVE 3 (ABI3) constitute components of the LEC1/B3 transcription factor network. Interactions of these genes result in temporal progression of overlapping B3 gene expression, resulting in aspects of embryo development that include storage product synthesis and seed maturation (Kumari et al., 2013). Three *VP1/ABI3-LIKE (VAP)* genes encode B3 proteins that include plant homeodomain-like and CW domains associated chromatin factors (Masaharu et al., 2007). *LEC1* was initially identified in a mutant related to cotyledon identity where leaf like characteristics were observed. These included the presence of trichomes not normally present on cotyledons. Embryonic disturbances were also observed such as abnormal hypocotyl elongation and changes in the meristem organisation (Meinke et al., 1994; West et al., 1994). *FUS3* mutants were also shown to exhibit early and late stage embryo phenotypes, once again the presence of trichomes and an inability to accumulate storage products (Baumlein et al., 1994; Keith et al., 1994). *FUS* comes from the Latin for purple due to the ectopic anthocyanin accumulation in *FUS3* mutants.

The genes causing the phenotypes were cloned and shown to encode two separate classes of transcription factors. *LEC1* encodes a Heme activated protein 3 (HAP3) sub-unit of the CCAAT- binding TF which is found almost exclusively in seed plants (Lotan et al., 1998). LEC2 and FUS3 are B3 domain transcription factors (Stone et al., 2001). *LEC* expression is almost exclusively observed through morphogenesis and maturation but drops off before germination.

The roles of *LEC* genes in embryogenesis are indicated by their gain and loss function phenotypes. When expressed ectopically, all three of the *LEC* genes cause cells in the post germination plant in both somatic and non-somatic tissue to adopt a maturation phase-like phenotype (Stone et al., 2001; Lotan et al., 1998). Plants which suffer a loss of function mutation in the *LEC* genes are intolerant to desiccation and show significantly lower levels of storage products such as starch (Ileda et al., 2006). *LEC1* and *FUS3* transcripts are preferentially localised to the protoderm (embryonic epidermis) in early embryogenesis, which is consistent with the presence of trichomes and anthocyanin exhibited in the *lec1* and *fus3* mutant phenotypes (Tsuchiyaet al., 2004). FUS3 under normal circumstances acts as a negative regulator of the anthocyanin-activating and trichome-activating *TRANSPARENT TESTA 1 (TTG1)* gene during embryo development. LEC2 is closely related to FUS3 and like LEC1 plays a role in cotyledon identity and storage product accumulation.

Ectopic expression of *LEC1* & *LEC2* has also been shown to trigger somatic embryogenesis in vegetative cells (Stone et al., 2001). The *LEC* genes are also necessary for complete, normal, morphogenesis of the zygote. The fact that they alone can trigger somatic embryogenesis can be seen as an indication that they play a role in totipotency (Harada, 2001) (Braybook and Harada, 2008).

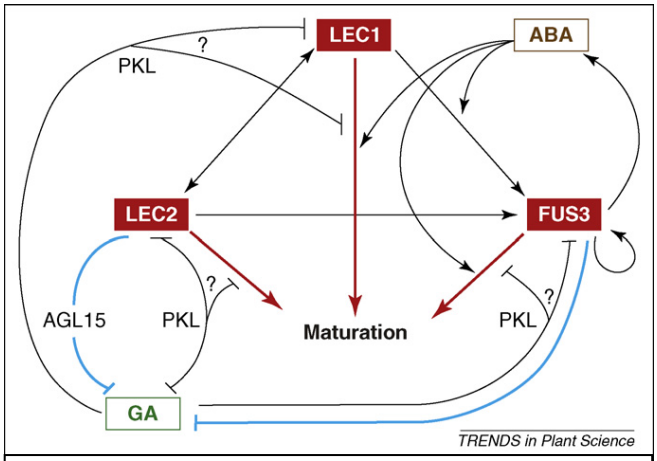



**Figure 2** *LECs* regulate maturation in embryogenesis by both regulating other *LECs, ABA and GA* directly and indirectly resulting in the formation of the mature seed. (Baybrook & Harada, 2008)

Activation of *LEC1, LEC2* or *FUS3* may also cause the accumulation of seed storage proteins and triglycerols (Lotan et al., 1998; Santos Mendoza et al., 2005) similar to the material seen in the *Arabidopsis turnip* (*tnp)* mutant discussed in further depth later. *tnp* is

characterised as a gain-of-function mutant of *LEC1* caused by an upstream promotor deletion. This results in ectopic expression of *LEC1,* but not of other *LEC* genes. Activation of *LEC1* in vegetative tissue causes several morphological changes including the loss of hypocotyl epidermal cell marker expression and loss of *SCARECROW* expression in the endodermis, the ectopic accumulation of starch and lipids, and the up-regulation of early and late embryonic genes. (Casson and Lindsey, 2006)

Maturation is a process that is exclusive to seed producing plants. It has been shown that the *LEC* genes are intrinsically linked to the accumulation of storage molecules and the induction of the maturation phase (Ileda et al., 2006). The LEC TFs directly target genes that cause the synthesis of the storage molecules by binding to the RY DNA motif (Dickenson et al., 1988). This motif is present in the 50 flanking regions of the embryo storage genes and the translation is rapidly upregulated on binding with the LEC TFs (Reidt, 2000). LEC2, FUS3 and ABI3 have conserved b3 DNA binding domains. ABI3 interacts with LEC TFs to help in the regulation of the storage protein genes during the maturation phase. ABI3 is the *Arabidopsis* orthologue of maize VP1 responsible for aspects of seed maturation control and prevention of premature germination (Suzuki and McCarty, 2008). *LEC1* expression appears to be dependent on the other *LEC* genes. This dependence is a complex process currently best described in Figure 2 above (Braybook and Harada, 2008).

*LEC2*, *FUS3* and *ABI3* expression are regulated by LEC1 although LEC2 may also activate *LEC1*. *fus3* and *lec2* mutants also show reduced *ABI3* expression (To et al., 2006; Baybrook and Harada, 2008). These mutants also show embryonic desiccation tolerance due to ABA signalling and accumulation of late-embryogenesis abundance-proteins (Hundertmark and Hincha, 2008). ABA signalling is a well known regulator of seed maturation where *abi3* is ABA insensitive (Finkelstein et al., 2002).

### 1.2.2 Hormonal interactions

Plant hormones such as abscisic acid (ABA) and gibberellic acid (GA) serve: physiological roles in seed maturation; timings controlled by stratification leading to germination; and seedling growth (Yamaguchi, 2007). After initiation of the maturation phase ABA levels show an increase and remain high until late in the phase where they drop off. After germination ABA levels remain low in the seedling (Yamaguchi, 2007). In direct contrast, GA levels are low during maturation but increase within the seedling after germination (Hays et al., 2001). ABA is thought to inhibit germination and hence it is the breakdown of ABA over the winter due to consistent cold conditions which allows plants to germinate in favourable spring conditions instead of brief warmer periods over the winter months.

The link between auxin and storage products is less easy to show, but it has been demonstrated that *LEC1* effects are dependent upon sucrose and auxin (Casson and Lindsey, 2006). Transgenic over expression of *LEC2* upregulates auxin biosynthesis by inducing *YUCCA* gene expression (Stone et al., 2008). *FUS3* is upregulated by auxin which also causes positive regulation of ABA levels (Gazzarini et al., 2004); but auxin also supresses *LEC2* expression. This further indicates crosstalk between: transcription factors, maturation, morphogenesis and auxin.

It is possible that *LEC* genes play a role in the ABA response. *lec1* and *fus3* mutants express occasional vivipary and ectopic *FUS3* causes an elevated level of ABA (Raz et al., 2001).

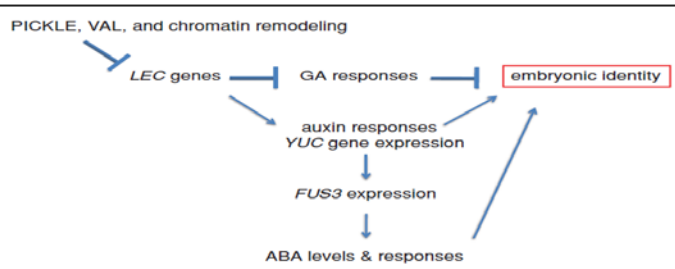



**Figure 3** LEC1/B3 genes are repressed in non-embryonic tissues by chromatin-associated factors, including PICKLE and VAL proteins. LEC genes appear to promote embryonic identity by repressing GA responses, promoting ABA responses, and may work via auxin. Taken from Kumari et al. 2013.

This is particularly interesting as *FUS3* and *LEC1* mediate accumulation of storage protein RNAs within the seed and are enhanced by ABA (Kagaya et al., 2005). LEC1 triggers the *FUS3* gene before the storage protein genes and before the expression is elevated by ABA. This suggests that ABA and *FUS3* could positively regulate each other.

Unlike auxin, GA synthesis is negatively regulated by FUS3 and LEC2. This is caused by the repression of the synthesis gene *GA3OX2* (Curaba et al., 2004; Gazzarini et al., 2004). LEC2 is responsible for directed activation of a catabolic gene *GA2OX6* resulting in a reduction of GA levels (Wang et al., 2004).

As previously stated, repression of the LEC1/B3 transcription factors is normal in vegetative tissue. This is thought to be in part mediated through PICKLE (PKL) via chromatin remodelling (Figure 2 & Figure 3). Mutants in *PKL* show storage oil accumulation and ectopic embryogenesis through depression of *LEC* genes (Ogas et al., 1997). The phenotype can be reversed via exogenous GA application or promoted via GA inhibitors. The *tnp* mutant also showed increased penetrance through the presence of GA inhibitors (Casson and Lindsey, 2006).

Cytokinins are also thought to have an effect but relatively little is known about their role in embryogenesis. *LEC2* and *FUS3* are both repressed by cytokinins (Casson and Lindsey, 2006).

### 1.3 The *tnp* mutant in *Arabidopsis*

#### 1.3.1 The structure of *Arabidopsis* and its use as a model system

*Arabidopsis thaliana* is a widely used model organism in plant research and development and was originally adopted for its usefulness in genetic experiments. As a result it was used as a standard reference in plant biology and is still used widely to this day. It was chosen as a model organism due to its small size and short generation time, paired with its ability to self-pollinate and create large amounts of seed (Scheres and Wolkenfelt, 1998). This allows large amounts of material to be grown within a short time frame which is extremely useful for genetic experiments. *Arabidopsis* was the first plant genome to be sequenced consisting of only ~26,000 genes (Arabidopsis Genome Initiative, 2000). The root has a largely fixed structure and is amenable to experimental manipulation making it a useful model for studying developmental processes such as patterning and hormonal response (Scheres and Wolkenfelt, 1998).

The anatomy of the *Arabidopsis* hypocotyl is well understood due to its wide use for physiological study of mechanism of cell elongation and the variety of biological events that constitute its control (Gendreau, 1997). The hypocotyl and root display radial organisation with concentric rings of cell files that are easily recognisable through their

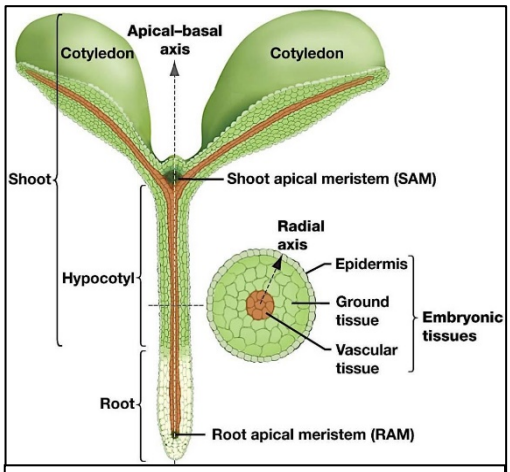



**Figure 4** Arabidopsis body plan, key components and tissues highlighted (Stufyblue, 2015)

morphology (Figure 4). From the outside in, these consist of: a) the epidermis, b) the ground tissue: consisting of parenchyma cells (generally used as filler cells), collenchyma cells for support (generally in regions of new growth), and sclerenchyma cells (containing thick lignified secondary walls providing support), c) the vascular tissue (consisting of the xylem

and phloem) which is located within the centre of the stem, surrounded by the ground tissue (Figure 4).

The hypocotyl is defined as the stem of the germinating seedling found below the cotyledons and above the radicle (Simpson and Weiner, 1989). The hypocotyl is formed during embryogenesis (Figure 1) and remains dormant in the seed until germination. After emergence of the radicle, the hypocotyl elongates without significant cortical or epidermal proliferation, however endoreduplication does occur (Gendreau, 1997). The growth pattern in the epidermis differs between light and dark: in the dark, epidermal cells elongate in a steep, acropetal spatial and temporal gradient whilst in the light, all epidermal cells elongate continuously during the entire growth period (Gendreau, 1997). Following photomorphogenesis, the true stem and leaves begin to develop leaving the hypocotyl as an intermediary zone between the stem and roots (Gendreau, 1997). In most species it becomes indistinguishable from the stem.

### 1.3.2 Identifying the *tnp* mutant

The *polaris* (*pls)* mutant is a defective gene encoding for a predicted small polypeptide required for root growth (Casson et al., 2002). *pls* was originally identified as a promoter-trap line sequence showing activity in the basal region of the embryo and in the seedling root tip (Topping et al., 1994). Whilst attempting to identify modifiers of *pls* expression, the trap line was mutagenized using T-DNA insertion. The mutated population was screened for altered expression of GUS. One mutant was identified where the *pls*-GUS was expressed abnormally at the hypocotyl root junction although expression was unaffected elsewhere. The hypocotyl root junction was also observed to be swollen and dense. The mutant appears to be dominant but shows incomplete penetrance. However, this may be to methylation-mediated gene silencing (Casson & Lindsey, 2006).

The *tnp* mutants exhibited a high degree of phenotypic variability and in some cases could be seedling-lethal. The strongest phenotypes showed complete hypocotyl structure replacement to that of a swollen structure. Weaker phenotypes showed some swellings throughout the hypocotyl, most commonly found at the hypocotyl root junction with dense greening cells (Casson & Lindsey, 2006). Examination of embryos of both the *tnp* and original *pls* showed no differences in the pre-germination structures. This suggests that the phenotype there develops post germination (Casson & Lindsey, 2006).

The surface patterning was explored using SEM. Epidermal cells appeared to be much smaller and flatter than the *pls* parent. Abnormal divisions occasionally occurred and, at the abnormal hypocotyl boundary, excessive cell elongation was observed (Casson & Lindsey, 2006). No patterning defects were observed internally but sections of the abnormal hypocotyls showed that the cells were lacking almost all vacuole. This looked structurally akin to storage tissue and staining with lugol and Fat Red showed a large presence of starch granules and lipids. This suggested altered cell identity.

### 1.3.3 The *tnp* mutant and *LEC1*

LEC1 is an important TF for both late and early embryogenesis and is normally restricted to expression in the pre-germination plant. *LEC1* expression has been shown to be repressed in vegetative tissue in part by PKL (Lotan et al., 1998). In the *tnp* mutant, ectopic expression of *LEC1* is observed due to the deletion of part of the gene promotor (Casson & Lindsey, 2006).

This deletion has been mapped to part of the regulatory section 436bp upstream of *LEC1* causing a 3,256 bp deletion. The subsequent ectopic expression of *LEC1* suggests that this promoter region was required for repression. The elevated ectopic expression was enough to cause the *tnp* phenotype but not sufficient to disrupt the formation of the embryo beforehand. The increased expression in vegetative tissue was shown to previously cause

embryonic-like morphology (Lotan et al., 1998) but in the *tnp* mutant lethality was rare and no development of ectopic embryos was observed (Casson & Lindsey, 2006). The hypocotyls of *tnp* did acquire some embryonic traits, the most phenotypically obvious being the activation of storage molecules such as starch and lipids.

It was also suggested that exogenous signalling molecules such as sucrose and auxin are mediated by LEC1 in the embryo (Casson & Lindsey, 2006). Both sucrose and auxin in the growth media were shown to play roles in the penetrance of the *tnp* phenotype. High concentrations of sucrose in the media may promote starch granule synthesis in the swollen hypocotyl. This could happen due to the overloading of the lipid storage pathways or through promotion of storage cell differentiation (Borisjuk *et al.*, 2002). Neither one of these suggestions has been shown to be convincingly conclusive but the idea could be followed further.

### 1.4 Anatomy of Storage roots

In many vegetables it is the root that acts as a storage organ and swells to a thickened state in order to sequester more storage molecules. There are many examples of this including carrots (*Daucus carota*), parsnips (*Pastinaca sativa*), dahlias (*Dahlia pinnata*) and many more. A small subsection of these root vegetables derive their swollen tissue from the hypocotyl as opposed to whole root. Examples of these include mostly *Brassicas* including the turnip (*B.rapa),* swede (*B.napus var.)* and kohl rabi *(B.oleracea)*. This is why their storage structure is much shorter and fatter than as seen in other root vegetables. In these storage roots or hypocotyls, the storage molecules are contained largely in the cortex, xylem or both (Biology Discussion, 2016).

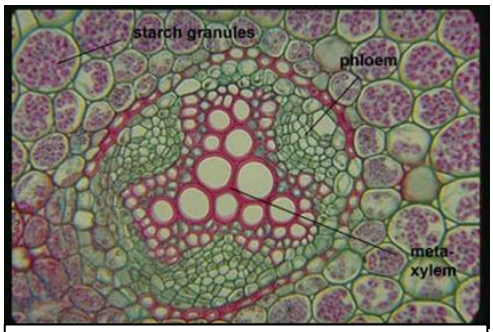



**Figure 5** A cross section of a dicot root, indicated is the central location of the xylem and phloem as opposed to the circular series seen within monocots (Leavingbio.net, 2016)

There is a difference in root tissue arrangement between dicots and monocots in the xylem and phloem. In dicots, the xylem and phloem are arranged in a singular central location (Figure 5), whilst in monocots they are arranged in a circular series around the root. All of the *Brassicas* examined were dicots. In *B. rapa* storage takes place largely in the xylem as the phloem and cortex are generally narrow (Biology Discussion, 2016). In the radish (*R. sativus)* storage also takes place within the xylem but is not limited to this region (Figure 6) whilst *D. carota* has equal storage between xylem and bark (Biology Discussion, 2016).

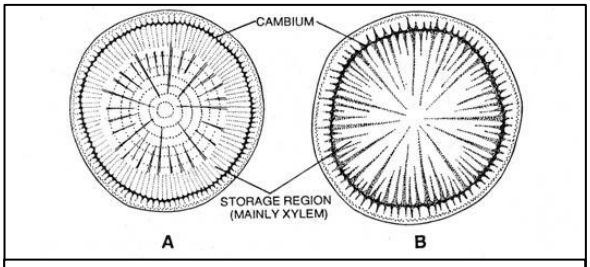



**Figure 6** Anatomy of storage roots A. Turnip Cross section B. Radish cross section. Indicated is the circular cambium region around the root. (B-discussion, 2016)

In *D. carota* the hypocotyl and the taproot form a singular fleshy structure. This organ has a large amount of storage parenchyma associated with the normal arrangement of tissue. This is formed through development of parenchyma in the phloem causing the root thickening. In *R. sativus* we see a similar arrangement with additions of parenchymal proliferation in the secondary xylem and pith (Figure 6). Within the parenchyma differentiation of vascular bundles are also observed (Biology Discussion, 2016).

### 1.5 *LEC1* in Brassicas

There has been little work to determine whether there is expression of *LEC1* in vegetative tissue of *Brassicas.* In this thesis I am hypothesising that an evolutionary link may exist between the formation of the hypocotyl vegetable and the recruitment of *LEC* orthologues. It was observed that *LEC1* was being expressed in the hypocotyl of *Brassica rapa* where the *tnp* like phenotype is observed (Tilley, 2010). *LEC1* expression was not only observed in the hypocotyl but throughout the whole plant. This raised questions as to why no embryonic

structures were observed elsewhere. It was not confirmed whether the *LEC1* expression was caused by an upstream deletion as seen in the *tnp* mutant in Casson & Lindsey (2006).

It is known that PKL is responsible for repression of *LEC1* in vegetative tissue via formation of a NuRD histone deacetylase complex. It is suggested that histones bound to the deleted promoter region could be targeted for deacetylation via the NuRD complex (Casson & Lindsey, 2006). This idea could be explored within the turnip tissue via analysis of the *PKL* gene.

The agro-industrial industry is constantly looking for new ways of viably producing proteins and oils. The ability to accumulate storage compounds such as starch and lipids that can be easily processed could be of great value (Wang et al., 2007). Vegetative *LEC1* expression in crop plants could facilitate the growth of crop plants and biofuels in the same system without the loss of the edible part of the crop to biofuel production. This waste is a large issue with first generation biofuels.

Genetic analyses of seed maturation regulators such as LEC proteins have shown that they can directly control the expression of seed storage proteins (Kagaya et al., 2005). It is, therefore, possible that once a full understanding of the regulatory mechanisms is obtained, any *LEC1* orthologues in other species could be genetically manipulated to produce novel biofuel crops.

### 1.6 Project Aims

It has been shown in *Arabidopsis* that overexpression of the embryonic gene *LEC1* causes accumulation of typical storage products such as starch, lipids and triacylglycerols in the hypocotyl (Casson & Lindsey, 2006). This forms a swollen turnip like structure independent of the root. It is hypothesised that these embryonic genes could be used as molecular switches in other mature plant species in order to activate morphogenetic and biochemical pathways normally only found in the embryo. This could have potential value in the biofuel

industry, allowing the design of novel crops that could sequestrate products that are much more easily/readily processed than current second generation biofuels such as cellulose.

The first objective is to grow a number of Brassica species. These will include: *Brassica rapa* (turnip - purple top), *Brassica rapa* (turnip - snowball), *Brassica oleracea* (kohl rabbi), *Brassica napus* (swede – helenor) and *Brassica napus* (oil seed rape – belinda); the last as a non-swollen hypocotyl control. A further hypocotyl derived vegetable will also be grown, not belonging to the Brassica genus: *Raphanus sativus* (radish – globe). This shares the same family as the other species, *Brassicaceae*. A time course shall be set up over an 8 week period and once more at 3 months. Each week a hypocotyl section from each species shall be stained for starch using Lugol, and fat, using Sudan Red. These shall then be imaged in order to create an anatomical series. In addition, RNA shall be extracted each week from the hypocotyl, leaf and root. The time course will show when and where expression is strongest and will allow comparison to the anatomical series. This is in order to address the following questions: Can we confirm *LEC* expression is present within Vegetative *Brassica* tissue as previously indicated? Like *tnp*, is expression limited to *LEC1* or are other *LECs* also expressed? If *LEC* expression is detected, do expression patterns correlate to the accumulation of starch and lipids within the *Brassica* hypocotyl?

Once the expression of *LEC1* has been determined, fatty acid analysis may be carried out on both *Arabidopsis* and *Brassica* tissue, both vegetative and seed. This is in order to provide a comparison of expected fatty acid profiles caused by *LEC* expression in the seed, and fatty acid profiles present within the hypocotyl of both *tnp* and *Brassica sp*. If similar fatty acids are present this could support the hypothesis that *LEC* expression plays a role in *Brassica* hypocotyl formation.

# 2 Methods and Materials

## 2.1 Materials

Most chemicals and materials were ordered from Sigma Aldrich unless stated otherwise in the text. For a full list of the plant, chemicals and experimental materials see Appendices 1-3.

## 2.2 *Arabidopsis* Growth and Maintenance

The wildtype *Arabidopsis thaliana* seeds used for laboratory controls were obtained from stores of Columbia (Col-0) originating from the Lehle seed line (Texas, US). The *tnp* mutant (Casson & Lindsey, 2006) was obtained from laboratory stocks. This was produced by the promoter trap line insertion. Twenty one insertion lines were available. Seedlings were grown within a Sanyo Growth Cabinet at 350 μmol $m^{-1}$ $sec^{-1}$ light intensity, 16 hour days.

### 2.2.1 Sterilisation

*Arabidopsis* seeds were sterilised for use in vitro using bleach and 98% ethanol under flow hood conditions.

The method:

- Under flow hood conditions add 1ml of 98% ethanol to an Eppendorf containing the seeds for 2 minutes
- Pipette off and add 1ml 20% Sodium hypochlorite & 0.1% Tween–20 for 15 minutes
- Rinse 3 times for 10 minutes in sterile distilled water
- Can be stored in Glycerol to aid plating later, store for 1 week in 4$^{o}$C to stratify.

### 2.2.2 Media and Plating

Murashige and Skoog (MS) Plant Culture Media was used as a growth standard. ½ MS 10 was used for normal bulking and MS 30 with the synthetic auxin 2,4-Dichlorophenoxyacetic acid (2,4 –D) 10nm was used to increase penetrance of the *tnp* phenotype. One litre of ½ MS 10 would contain 2.2g of MS salts, 10g of sucrose and 5g of agar. The combination of 2,4-D

and elevated sucrose was shown to increase penetrance of the phenotype by 90% (Casson & Lindsey, 2006).

Method for 2 litres:

- A large measuring beaker was filled with 1 litre of deionized water and magnetic flea
- The required level of MS salts was added to the beaker (4.4g for 1/2, 8.8g for full strength)
- Sucrose was then added ( 10 $g.L^{-1}$ for MS 10)
- Using a magnetic plate, the salts were stirred and dissolved in the deionized water.
- Using a pH meter 0.5M KOH was added until a pH 5.8 was obtained
- The mixture was decanted into suitable containers, agar added and autoclaved

Media was melted for use in the microwave, 50ml required for each 120mm x 120mm plate. Addition of 2, 4–D to media was carried out by first making up a 10nM stock in ethanol using serial dilutions. The media was then decanted into 50ml tubes before decantation into the sterile plates. This way the 2, 4-D was added to the media when required to prevent its breakdown in storage.

### 2.2.3 Growth Conditions

For hypocotyl assays, seeds were placed using a 20µl pipette tip in two lines across a $120mm^2$ petri plate. These were sealed with Micropore tape and maintained in a Sanyo growth cabinet ($22^oC$, 18 hour photoperiod).

For bulking, seedlings were first germinated on ½MS 10 and transferred to soil for three weeks in a growth room ($22^oC$, 18 hour photoperiod). Once the rosette was formed and the stem of suitable length, aracons and tubing were added. The plants were dried for a month before seeds were harvested. Soil was treated with Intercept to prevent infection and blackfly.

## 2.3 *Arabidopsis* Analysis

### 2.3.1 Phenotype Screening

Twenty one insertion lines were examined for best expression of the swollen hypocotyl phenotype. After 7 days of growth on vertical square plates with MS 30, all seedlings were imaged with a millimetre scale (Epson 1680 pro flathead scanner). Hypocotyl diameters were measured at the widest point and compared to the wild type to ensure penetrance was being observed. Each assay was repeated three times with at least 30 individuals on each plate. Images were analysed using the program Image J.

### 2.3.2 Hypocotyl assays

To ensure the phenotype was indicative of the original *tnp* mutant, Lugol was used to stain for starch at the hypocotyl root junction.

Method:

- The seedlings were removed from the growth media and placed on a glass slide. If they were grown vertically Lugol was directly applied to the Petri dish. This slightly yellowed the agar.
- The seedlings were left for 5 minutes in the Lugol.
- The Lugol was removed using absorbent tissue and the seedling rinsed with water, then left within the water for 10 minutes.
- If it was still hard to distinguish the dark blue from the absorbed Lugol background, Histoclear was used to reduce the background noise by soaking for 10 minutes.

Percentage penetrance was calculated using the proportion of those exhibiting the phenotype against the total number of each insertion line.

### 2.3.3 Seed assays

Sterilised seeds were plated onto square Petri dishes and imaged with a millimetre scale (Epson 1680 pro flathead scanner). The length and width were measured for each seed using Image J. In excess of 100 undred seeds of the mutant and wildtype were used for this assay.

An estimated volume was calculated for comparison using the equation $V = 4/3 \times \pi \times L \times W^2$ where L is length and W is width.

### 2.3.4 Embryo assays

Embryos were obtained from bulking plant lines. Siliques were taken whilst still green as opposed to dried; this was due to an inability to extract embryos from dried seeds consistently. The Siliques were sliced open using a needle and seeds transferred to a glass microscope slide. A small quantity of water was added to the seeds and a glass cover slip placed over the top. Using a Leica stereomicroscope the seeds could be viewed whilst pressure was applied to the cover slip using forceps. By pressing in the vicinity of the seeds this allowed the embryos to exit the seed case unharmed.

Staining Method:

- The embryos were submerged in Lugol for 5 minutes
- The Lugol was removed, and the embryos submerged in water for 5 minutes
- The water was removed , and the embryos submerged in Histoclear for 10 minutes
- Whilst submerged in Histoclear, the embryos were viewed.

The embryos absorbed the Lugol much more readily than the 7 day seedlings making the use of Histoclear mandatory. Images of the embryos were taken using a Qi Cam utilising Open lab software.

## 2.4 Brassica Growth and Maintenance

The seeds that were used are commercially available online:

| | |
|---|---|
| • Johnsons turnip seeds | *Brassica rapa* – Varieties 'Purple top' and 'Snowball' |
| • Fothergill turnip seeds | *Brassica napus var. napobrassica* – Variety 'Helenor' |
| | *Brassica oleracea* – Variety 'Olivia' |
| | *Raphanus sativus* – Variety 'Globe' |

Oil seed rape seeds were obtained from lab stores and originally obtained from Newcastle University, Belinda line. When *Brassica napus* is stated in text presume it is referring to *var. napobrassica* (Swede) unless stated otherwise

### 2.4.1 Germination and Growth Conditions

To germinate, the seeds were placed in circular Petri dishes with moist filter paper and stratified for 7 days at 4$^{o}$C in the dark. Once stratified the Petri dishes were transferred to a growth Sanyo until germination. Once germinated seedlings were transferred to individual 10cm plastic pots containing John Innes No.3 compost and transferred to an illuminated greenhouse (26$^{o}$C, 18 hour photoperiod).

## 2.5 Brassica Analysis

### 2.5.1 Sectioning

Sections were used for Starch, Fatty acid and Lignin staining. Whilst the plants were young; 1 – 3 weeks; thin sections could be obtained using a Stanley Knife. Once the plants and hypocotyls got thicker a mandolin or chef's knife was used to obtain sections.

### 2.5.2 Staining

Starch was stained for using Lugol

Method:

- The section was submerged in Lugol for 5 minutes, in larger sections surface tension was used to avoid submerging the whole section and simply covered the top
- The section was then rinsed and submerge in water for 10 minutes.
- Finally the section was imaged using Qi Cam

Neutral Lipids were stained for using Fat Red 7B (Brundrett et al., 1991)

Stock 0.1%

- 50mg of Fat Red was dissolved in 25ml of PEG-300
- This was incubated for 1 hour at 90$^{o}$C and allowed to cool
- 25ml of 90% Glycerol was added and vortexed

Method:

- The section was submerged in Fat Red for 5 minutes, in larger sections surface tension was used to avoid submerging the whole section and simply covered the top
- The section was then rinsed and submerge in water for 10 minutes.
- Finally the section was imaged using Qi Cam

Lignin was stained for using Phloroglucinol

Stock 0.5%

- 5g of powder per litre was dissolved in 95% alcohol
- A working stock was created by dissolving 4 parts Phloroglucinol to one part HCl

Method:

- Section were submerged in working stock solution for 10 minutes
- In the presence of lignin, a bright red stain was observed.

Once the hypocotyls would no longer fit under the microscope a camera and tripod was used in its place. Cannon 7D, 50mm Lens, no Flash, f3.0, ISO 400.

### 2.4.3 Tissue collection and storage

Tissue was divided into three sections: hypocotyl, leaf and root. For samples from week one and two, whole seedlings were used for RNA extraction. This was due to difficulties in growing large enough quantities of the individual tissues to allow adequate yield. Tissue was cubed into $2cm^3$ blocks, foil wrapped and flash frozen in liquid nitrogen. This was stored at -$80^oC$ until ground for use.

## 2.6 Primer design

Primers were designed using bioinformatics information obtained from online resources. *LEC1*, *LEC2* and *FUS3* sequences were acquired in the *Arabidopsis* genome from The *Arabidopsis* Information Resource (*Arabidopsis*.org, 2016). These sequences were then blasted against both *Brassica* and *Raphanus* cDNA databases (Brassicadb.org, 2016 & Mitsui et al., 2015). In the Brassicas, each gene orthologue across all species were aligned against the *Arabidopsis* cDNA sequence using the bioinformatics program ApE (v2.0.49, January 29 2016). Regions of high homology were selected manually across all orthologues, and primers for these regions were designed using the online resource BLASTN (Blast.ncbi.nlm.nih.gov, 2016). The regions the primers bound were then tested for

**Table 3.** Primers designed over regions of high homology between *Col 0* and *Brassica* orthologues

| **Primer** | **Sequence** | **cDNA product length** |
|---|---|---|
| BLEC1F | CTCGTGAGCAAGACCAATACAT | - |
| BLEC1R | CACGGTACCGGTTAATGAACAC | 275 |
| BLEC2F | ACGCAAACTTTGTCCAAGAACT | - |
| BLEC2R | TCTGTTGATCCTGGCCATCT | 280 |
| BFUS3F | CTGTGCCTCTTCTGGGTGTT | - |
| BFUS3R | AGGCCAGAACCTGTACTTGA | 206 |

**Table 4.** Primers designed over regions of high homology between *Col 0* and *Raphanus* orthologues

| **Primer** | **Sequence** | **cDNA product length** |
|---|---|---|
| BLEC1F | TCAAGAATGCGTCTCCGAGT | - |
| BLEC1R | AGTACCGACCACCACCCATA | 341 |
| BLEC2F | GCCCTTTTCCTCTTCTAACGC | - |
| BLEC2R | CGCCTTCTTCCTGTTGATCCT | 310 |
| BFUS3F | CCCTTTTCCTCTTCTAACGCA | - |
| BFUS3R | ATCATCGCCTTCTTCCTGTTGA | 314 |

specificity using the same recourse. *Raphanus* primers were designed in an identical way but aligned only against orthologues of *Raphanus sativus* and *Arabidopsis thaliana*. This led to the following primers being designed (table 3 & table 4), cDNA alignment for each design is provided in Appendix 4.

### 2.7 RNA extraction

RNA was extracted from each sample Helenor' (*Brassica napus*), 'Purple top' (*Brassica rapa*), 'Snowball' (*Brassica rapa*), 'Olivia' (*Brassica oleracea*) and 'Globe' (*Raphanus Sativus*). Each extraction required ~100mg of ground tissue and no more than 120mg. Smaller samples such as tissue from 1 or 2 weeks of growth were ground in an Eppendorf under lysis solution. Larger tissues were ground in heat sterilised mortar and pestles under liquid nitrogen until a fine powder was achieved.

The RNA extraction was carried out using the Sigma 'Spectrum™ Plant Total RNA Kit', the protocol for which was provided by the supplier. Hypocotyl tissue was treated as a starch storage organ and was therefore incubated at room temperature instead of 56$^{o}$C. When binding RNA to the column, protocol B was used for hypocotyl tissue, leaf and root followed protocol A. Protocol B used a smaller quantity of binding solution and was recommended for leaf with normal water content (such as grape and tomato leaves), and starch storage organs. This allowed higher concentrations of RNA to be extracted from difficult tissue and yield usable levels for cDNA synthesis, the exact protocol can be viewed online. An on-column DNA digest was performed using the Sigma 'DNase I digest set' as instructed in the protocol. The final elution RNA concentration was measured using a Nanodrop ND1000 Spectrophotometer (ThermoFisher Scientific, Hemel Hempstead, UK). If RNA concentrations were less than 100ng.$\mu l^{-1}$ and there was insufficient tissue remaining to repeat an extraction, the Eppendorf Concentrator 5301 was used to concentrate the RNA to a sufficient level. Three biological repeats were carried out for each tissue from each sample.

## 2.8 cDNA Synthesis

For cDNA synthesis concentrations of RNA were standardised to 100 ng/µl. Each reaction used 3µg of RNA in 20 µl of solution, reaction mixes were made up according to Table 5. The enzyme used was Superscript III First-Strand Synthesis System (Invitrogen Ltd, Paisley, UK).

**Table 5.** Reaction Mixes used for cDNA synthesis

| **RNA Mix** | **Volume per reaction (µl)** |
|---|---|
| RNA | 10 |
| OligoDT20(50µM) | 1 |
| dNTPs(10mM) | 1 |
| Total | 12 |
| **cDNA synthesis mix** | |
| 5x FS Buffer | 4 |
| 0.1M DTT | 1 |
| RNAse out | 1 |
| Superscript III | 1 |
| Incubated RNA mix | 12 |
| Total | 20 |
| RNase H | 1 |

**cDNA synthesis method:**

1. The RNA mix was incubated at 65$^{o}$C for 5 minutes, then cooled on ice for 1 minute
2. 13µl of Master mix was added to each tube and mixed well.
3. This was incubated at 50$^{o}$C for 50 minutes
4. Then the temperature was increased to 85$^{o}$C for 5 minutes
5. 1µl of RNase H was added to the mixture
6. This was incubated at 37$^{o}$C for 20 minutes
7. ¼ dilution was used for PCR strength, this was stored at -20$^{o}$C long term

## 2.9 PCR

The cDNA synthesis product was tested using standard PCR amplification of ACT2, Primers seen in table 6.

**Table 6.** Actin primers designed in regions of 100% homology in *Brassica napus* & *Brassica rapa* 1 intron detected. (*Unpublished University of Durham 2010).*

| **Primer** | **Sequence** | **cDNA product length** |
|---|---|---|
| BNACT2F | GTGACAATGGAACTGGAATGGT | - |
| BNACT2R | GCCTGGATAGCAACATACATGGCA | 385 |

Standard PCR was also used to test the primers to be used in qRT-PCR (Tables 3 & 4). Each reaction was set up using the following reaction mixes (Table 7) and the PCR cycling conditions (Table 8) as stated according to the standard MyTAQ DNA polymerase protocol (Bioline). All PCR reactions were performed using an Applied G-Storm GS1 PCR machine.

| **Table 7. PCR reaction mix** | |
|---|---|
| **PCR mix** | **Volume per reaction (µl)** |
| 5x MyTAQ Reaction Buffer | 4 |
| Forward Primer (20µM) | 0.5 |
| Reverse Primer (20µM) | 0.5 |
| MyTaq DNA polymerase | 0.1 |
| Water ($dH_20$ MilliQ) | 14.5 |
| cDNA template | 0.4 |
| Total | 20 |

| **Table 8. PCR cycling conditions** | | | |
|---|---|---|---|
| **Step** | **Temperature $^oC$** | **Time** | **Cycles** |
| Initial Denaturation | 95 | 1 min | 1 |
| Denaturation | 95 | 15 seconds | 35 |
| Annealing | 55 | 15 seconds | |
| Extension | 72 | 10 second | |

## 2.10 Gel Electrophoresis

Agarose gel (2%) was used to run PCR products under electrophoresis. This was made up using 1x TAE buffer, agarose and ethidium bromide (5µl/100ml). Each well was loaded with 10µl of sample (containing 5x loading buffer) and a separate well containing 5µl of Bioline Hyperladder for fragment size determination. The gel ran at 110 volts for 30 minutes. Once the gel had run, the results would be viewed using an UV illuminator (Syngene, 'InGenius L').

## 2.11 qRT –PCR

The Quantitative real-time polymerase chain reactions (qRT-PCR) were carried out using SensiFAST™ SYBR® Lo-ROX Kit (Bioline) on a Rotorgene 3000 (Qiagen). The reaction mix for each run is specified in table 9 and used primers described in tables 3,4 & 6.

**Table 9.** qPCR mixture volumes

| **Component** | **Volume for 1x reaction (µl)** |
|---|---|
| Forward Primer (20µM) | 0.4 |
| Reverse Primer (20µM) | 0.4 |
| cDNA template | 1 |
| Sensifast SJ | 10 |
| Water ($dH_2O$ MilliQ) | 4.2 |
| Total | 20 |

Values were calculated using three technical repeats for each sample. Gene expression levels were calculated relative to the amplification of the reference gene Act2 (AT5G1570) using the Rotorgene Q series software v1.7. Act2 was chosen due its stability relative to stress conditions throughout tissue type (Czechowski et al., 2005). Reference gene stability was

tested by comparing expression levels between samples; the amplification specificity was checked using melt curve analysis.

### 2.12 Fatty Acid Extraction

Fatty acids were extracted from a range of samples representing different stages in the life cycles of both *Arabidopsis* and *Brassica sp*. The protocol differed between seed and adult tissue extractions. For both protocols the standard was calculated at 10% of the overall fatty acid content.

#### 2.12.1 Seed extraction

Equipment used must be glass due to chloroform use; this includes glass pipettes and vials. Each reaction used 20 *Arabidopsis* seeds or 2 *Brassica seeds.* Large numbers of seeds were counted and weighed on mass to calculate an average seed weight.

The protocol used was as follows:

1. A standard was made up (10% of fatty acid content), this was calculated using an estimated 40% fatty acid content by weight of the seed; this resulted in 1nM/1µl concentration.
2. The seeds were placed into glass test tube with a Teflon lid, an alternative to Teflon could be used to prevent volatiles escaping. Thin forceps were used for transport of *Arabidopsis* seeds.
3. 10µl of standard was added to the seeds
4. 500µl of Methanolic HCl was added and vortexed, at this point toluene can be added to increase yield
5. Ensuring the lid was screwed on tight the mixture was incubate at 80$^{o}$C overnight.
6. Allowing the mixture to cool before next steps. The Gas Chromatography vials were prepared.
7. 500µl of 1% KCl was added to the seed-methanolic HCl mix
8. 150µl of 1% Hexane was added and shook to observe the 2 phases

9. This was centrifuged for 10mins at 0.5-1 RPM (x1000)
10. The thin layer of hexane from the top phase was extracted carefully in order to pipette nothing else. If this occurred, the tube was re-centrifuged and the phase re-extracted
11. Samples were run using Gas Chromatography Mass Spectrometry, see Appendix 5

### 2.12.2 Tissue extraction based on Bligh &Dyer, 1959

The same equipment was used as in the seed extraction protocol.

1. 1g of fresh tissue was weighed for maceration
2. Maceration took place under liquid nitrogen or in 1ml of 2:1 Chloroform:methanol
3. The ground tissue was decanted into a Teflon topped glass tube, the mortar and pestle rinsed with a further 1ml of Chloroform:Methanol to ensure all material was removed. If tissue was ground under liquid nitrogen 2ml of 2:1 Chloroform:methanol was added directly to the tube.
4. This was centrifuged at 1000rpm for 10 minutes
5. If the top layer of supernatant was still coloured a further 1-2ml of Chloroform was added and the mixture centrifuged for a further 10 minutes.
6. The BOTTOM phase was extracted into a fresh tube and all liquid evaporated off using nitrogen gas.
7. This was re-dissolved in 200µl Chloroform: Methanol and spun down if debris was present
8. 20µl of the mixture was aliquoted into a fresh tube.
9. 500µl Methanolic HCl and 20µl of standard was added
10. The Teflon cap was screwed on tight and incubate overnight at 80$^{o}$C
11. Allowing the mixture to cool, 500µl 1% KCl was added
12. Followed by 200µl of Hexane
13. This was centrifuged for 10mins at 0.5-1 RPM (x1000)

14. The thin layer of hexane was extraced from the top phase being careful to pipette nothing else. If this did occur the tube was re-centrifuged and re-extracted
15. The Sample was run using Gas Chromatography Mass Spectrometry, See Appendix 5

### 2.13 Statistics Analysis

All statistics were calculated using either IBM SPSS Statistics 2.0 or Microsoft Excel 2013

# 3 Results

## 3.1 *tnp* mutant in *Arabidopsis*

### 3.1.1 Abnormal starch as an indicator of *tnp* penetrance

The original *tnp* mutant was discovered in the *polaris* mutant (*pls)* background, the *pls* mutant itself has a strong phenotype typical of the ethylene pathway (Casson & Lindsey, 2006). *tnp* was found to contain a mutation in *LEC1*, as an upstream deletion of the promoter region that is thought to have released suppression of the gene in seedlings. The original *tnp* mutant showed variable penetrance that was shown to improve in the presence of the synthetic auxin 2, 4-D and increased sucrose concentration (Casson & Lindsey, 2006). To better understand the *tnp* gain of function mutant phenotype without the *pls* background, the *LEC1* sequence was inserted without the native promoter region but using the CaMV35 promotor, to allow ectopic expression without usual repression in the adult tissue (Casson, unpublished). These lines were grown and compared to Col-0 to determine which insertion produced the best penetrance of the *tnp* phenotype. Penetrance of the mutant was determined by the presence or absence of starch at the hypocotyl-root junction which was easily observed by addition of Lugol solution (Figure 7).

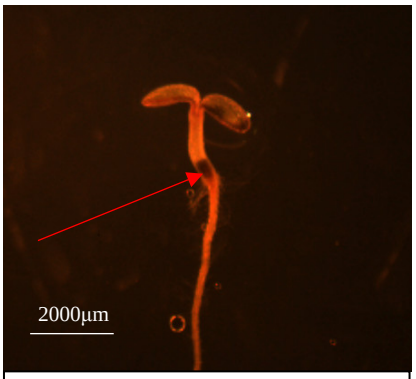



**Figure 7** 7 day old *tnp* seedling grown on ½ MS 30 & 10nm 2,4D stained with Lugol solution. Arrow indicates a strong presence of starch within the hypocotyl which is indicative of the *tnp* phenotype.

**Table 10.** Percentage penetrance of the *tnp* phenotype on various insertion lines when grown on agar vs phytogel in order to achieve maximum usable samples. *Col 0* used as a control.

| | **½ MS 30 + 2,4 D** | | | |
|---|---|---|---|---|
| | **Agar** | | **Phytogel** | |
| **Line** | ***Tnp*** | **Fatal** | ***Tnp*** | **Fatal** |
| Tnp 21 | 87.5% | 12.5% | 55.5% | 12.5% |
| Tnp 20 | 85.7% | 14.3% | 70% | 0% |
| Tnp 12 | 57% | 7% | 6.25% | 37.5% |
| Tnp 8 | 70% | 0% | 33.3% | 33.3% |
| Tnp 7 | 77.7% | 0% | 10% | 30% |
| Col 0 | 30% | 0% | 20% | 0% |

The abnormal presence of starch is stained black and indicated by the arrow at the hypocotyl root junction in Figure 7, normally no staining would be observed in this location.

Growth on agar medium produced a stronger phenotypic response than on

phytogel as a thickening agent in all cases (Table 10). The combination of higher levels of sucrose, 30 $g.L^{-1}$ as opposed to 10 $g.L^{-1}$, and the addition of 2, 4-D appeared to cause penetrance of relatively high levels across all lines as seen in Casson & Lindsey, (2006). It also indicated the best lines to use for future experiments (Tnp7, Tnp20 & Tnp21). The lethal *tnp* phenotype was still observed in most cases, but when agar was used as a thickening agent instead of phytogel, the levels were reduced to below 15% making it manageable for assays. Therefore line 21 was selected for use in further experimentation, unless otherwise stated. This was due to the highest percentage of penetrance of the *tnp* phenotype.

### 3.1.2 Induced *tnp* phenotype in *Arabidopsis*

Introduction of synthetic auxin into the medium increased penetrance of the *tnp* mutant (Table 10) and also increased the incidence of starch accumulation at the hypocotyl-root junction in *Col 0* from 5% in the wildtype to 30% (n=132). When stained with Lugol the hypocotyl appeared significantly less darkened than the original *tnp* mutant (Casson and Lindsey, 2006) but is still noticeable (Figure 8). Interestingly there is also starch present within the cotyledons which is not normally observed in the *tnp* mutant (Casson and Lindsey, 2006). These locations are indicated in Figure 8 by the arrows highlighting the darkened regions where starch has been shown to be present.


**Figure 8** 7 day old *Col 0* seedling grown on ½ MS 30 & 10nm 2,4D stained with Lugol solution. Arrows indicate locations of starch staining which is not usually observed when the wild type is grown in the absence of 2,4 D.

2, 4-D is already known to produce a shortened root phenotype which was observed (students t-test, $p < 0.001$, d.f. = 67). GENEVESTIGATOR, a bioinformatics program, was used to investigate a possible auxin mediated upregulation. This uses a database of thousands of manually curated, well described public microarray and RNAseq experiments

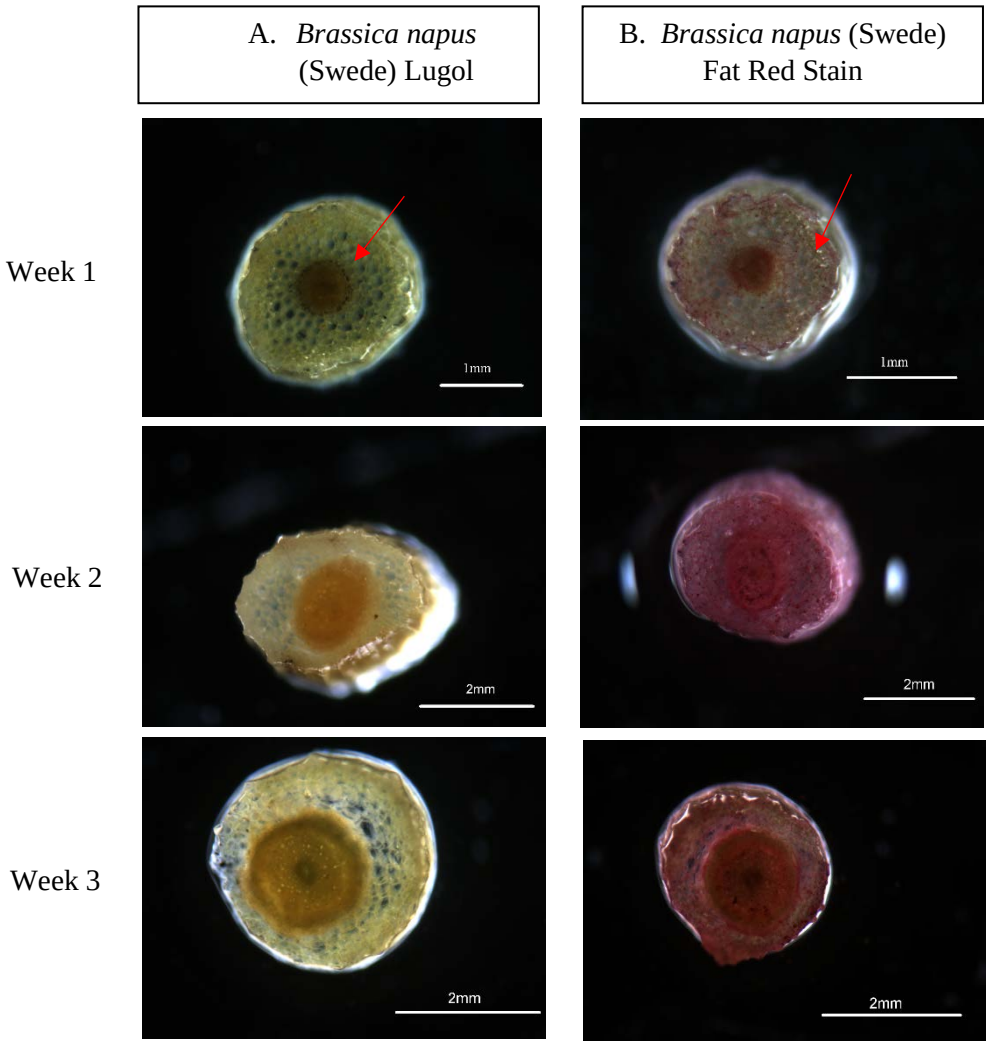
and nicely visualizes gene expression across different biological contexts such as diseases, drugs, tissues, cancers, cell lines or genotypes. The high diversity of curated experiments allows GENEVESTIGATOR to project genes against a broad spectrum of reference profiles and datasets. This allowed investigation into whether auxin or its orthologues have been demonstrated to upregulate LECs in previous microarray data. There appeared no indication that this was occurring in *LEC1*, *LEC2* or *FUS3* in any auxin treatment, synthetic or non-synthetic. This suggested no evidence for a role in auxin-mediated penetrance of the *tnp* phenotype via the upregulation of these genes

PCR was performed on the starch producing *Col 0* but there was no evidence that *LEC1* or *LEC2* had been upregulated, data not shown.

### 3.2 Histochemical Staining

Much like the *tnp* mutant observed in *Arabidopsis thaliana,* storage compounds in *Brassicas* are sequestered in the hypocotyl. This is fairly unusual in most vegetables where the hypocotyl is relatively small and normally indistinguishable from the stem. The edible portions are normally derived from sections such as the fruit (e.g. tomatoes), the root (e.g. carrots), tubers (e.g. potatoes) or the bulb (e.g. onions).

#### 3.2.1 Brassica Sections




*Continued next page*

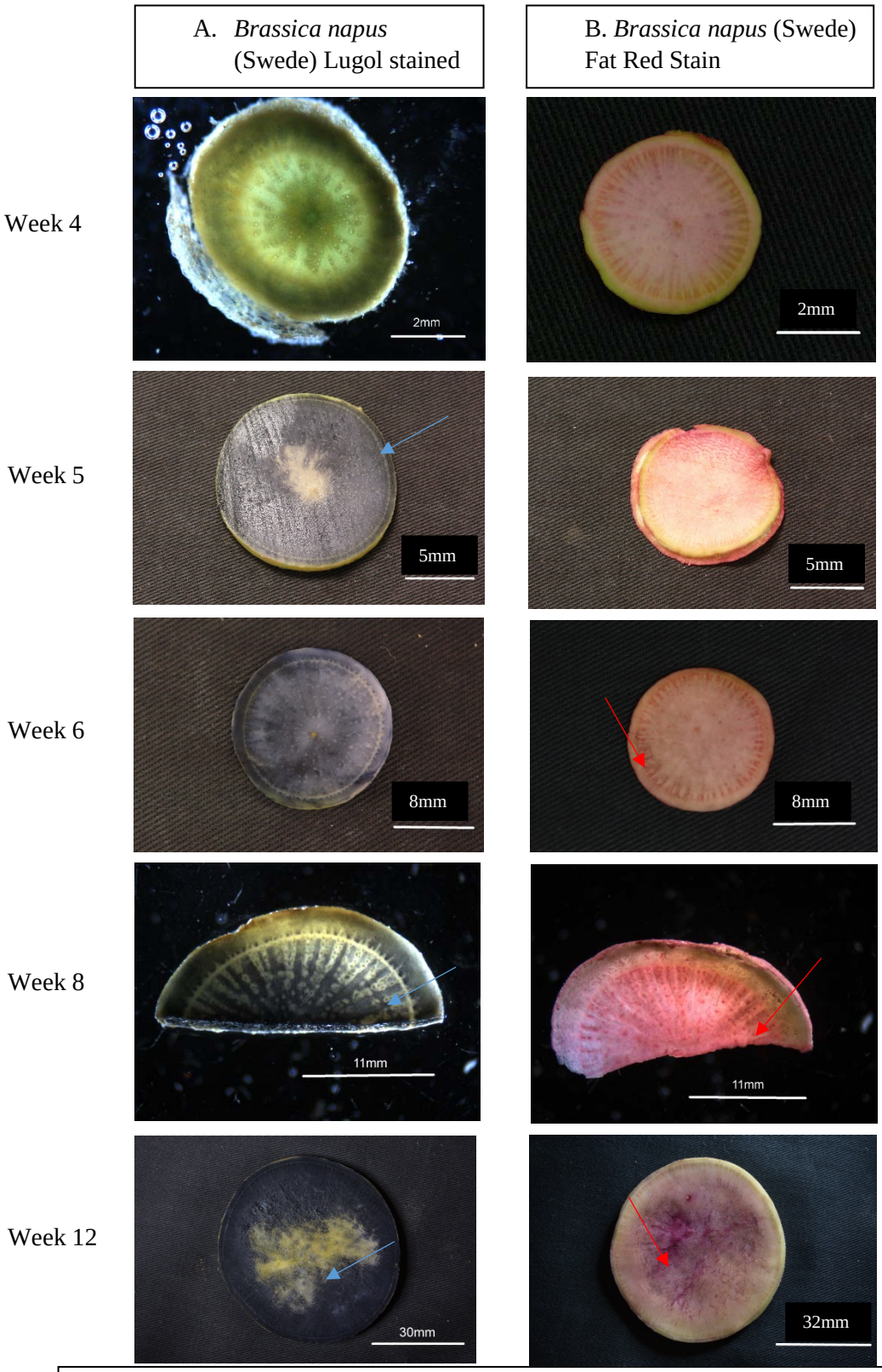



**Figure 9** Sections of *Brassica napus* stained with Lugol for starch (A) and Sudan Red for lipids (B). The time course takes place over a 12 week period showing weeks 1-6, 8 and 12 where 12 represents a mature specimen. Week 1 represents 7 days after germination had been observed; subsequent weeks are also based from germination. Methods for sectioning and staining are explained in the methods sections. Red arrows indicate locations of starch/lipid presence; blue arrows highlight areas of absence

A time course, through multiple *Brassicas* growth cycles, was carried out to determine if similar compounds that are observed within seeds for drought protection and dormancy were present in *Brassica* hypocotyls. It was explored whether *LEC* expression levels in *Brassicas* correlated with points in the growth cycle where these compounds are observed in significant quantity. It is known that *LECs* are intrinsically linked to these storage compounds expression in seeds (Ileda et al., 2006) but, not if a similar pathway is responsible for the post germination hypocotyl development in *Brassicas*. Samples for both RNA extractions and sections for staining were carried out simultaneously. The natural colouring of *Brassica napus* contains neither black, nor red. Any colouring present can therefore be assumed to be from histochemical positive staining. The unstained section are a greenish white in colour (Figure 10).

**Figure 10** Unstained *Brassica napus* section ("Neeps And Tatties (Or, Scottish Root Vegetable Extravaganza)")

Figure 9 shows the time course for one of the *Brassica sp.* examined (*Brassica napus)* as a representative example. The other sectional series may be seen in the appendices (Appendix 6). It can be seen that one week after germination there is a small population of starch granules (Figure 9, A, Week 1) located around the stele of the hypocotyl, indicated by the red arrow. These smaller granules are no longer obviously present in subsequent weeks 2 or 3. This is thought to be a remnant from the embryo and is explored in more detail later (Section 3.3).

Starch accumulation appears to take place in large quantities by week 5, (Figure 9, A, Week 5) prior to this there is no starch staining present in weeks 2-3 even around the stele as

observed in week 1. There is indication that accumulation begins at week 4 beneath the epidermis in the ground tissue of the hypocotyl, the location of which is indicated on the figure with an arrow (Figure 9, A, Week 4). It is also at this time point that an increase in hypocotyl swelling is seen, compared to the stem elongation and thickening. Weeks 5-8 show a very similar staining profile, with starch being contained mostly within the xylem in large uniform quantities; this is represented by the large amounts of black staining seen within Figure 9 at the respective time points. Externally to the xylem is an outer ring of unstained cambium cells (indicated on week 5 by the blue arrow) and further staining between the cambium and exodermis (Figure 9, A, Week 5-8). The light microscopy image of week 8 shows that it is not the entire xylem stained by Lugol, but contains sections of unstained material indicated by the blue arrow. The major morphological changes within this time period are a large increase in hypocotyl volume from a width of 1cm to 6cm and an increase in size of the rest of the plant. By the time the plant is mature, a non-uniform central section was observed that does not stain positive for starch (Figure 9, A, Week 12) (indicated by the arrow). This is possibly a structural cellulose modification to support the larger structure which is explored in greater detail later on (Section 3.2.4).

Lipid staining was much harder to quantify due to the ambiguous nature of the stain. The overall staining pattern within the hypocotyl appeared to mirror that of the starch, but not always on a temporal level. Initially at week 1 we see a small amount of staining around the periphery before hypocotyl expansion is observed, indicated by the arrow (Figure 9, B, Week 1). It is possible, like the starch granules observed at the same time, that these could be an embryonic remnant. The lower resolution images of the larger tissues (Figure 9, A, Weeks 5,6 & 12), too large for the microscope, does not give an indication of continued accumulation. What was observed was a presence of lipids in weeks 2-3 throughout the hypocotyl before starch was observed - see the large amounts of red staining. (Figure 9, B Weeks 2-3). There was no obvious change in patterning between weeks 4-8 where lipids are present throughout the xylem, concentrated on the inner side of the cambium (Figure 9, B

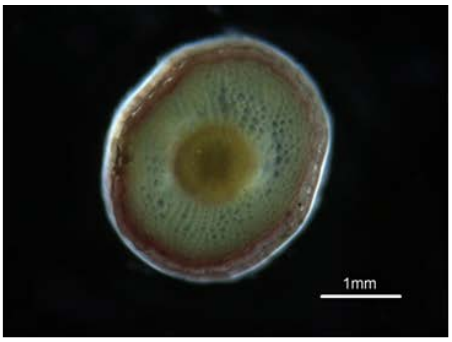
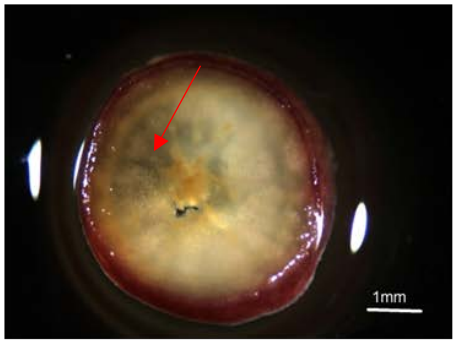
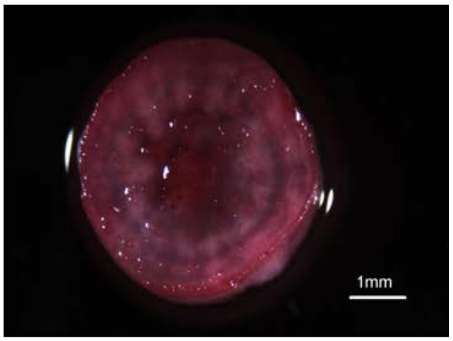
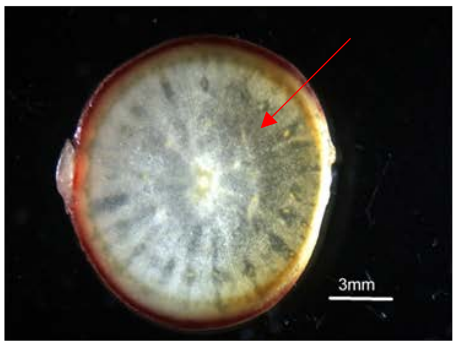
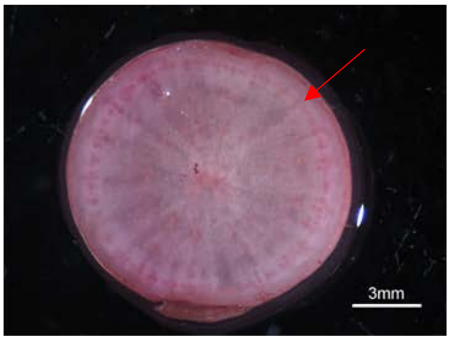
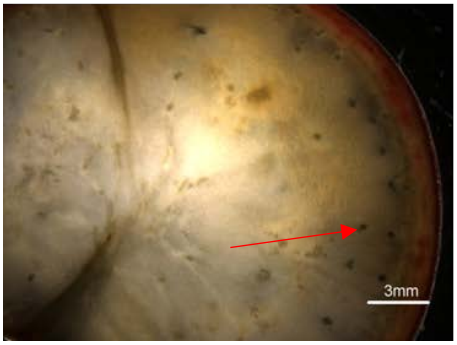
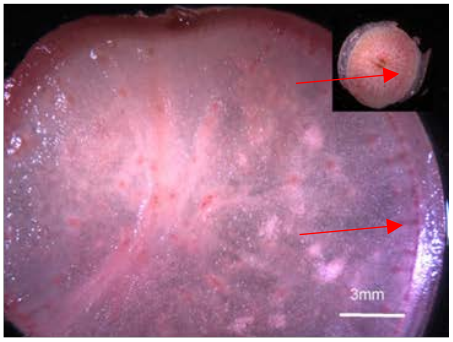
Weeks 4-8). Week 8 shows a small section of the hypocotyl once again under the bright field, illustrating the similar spatial staining pattern to that of starch (Figure 9, B Week 8). Unlike starch, there is only a minimal amount of stain present between the cambium cells and the exodermis. By the time the plant is mature, the staining pattern appears to move closer to the centre of the hypocotyl but it was not possible to determine whether the absent zone, as seen with the starch, was mirrored (Figure 9, B Weeks 12).

The staining series in the other *Brassicas* are largely similar first showing xylem expansion between week 1 and week 4. Hypocotyl expansion is observed by week 5 in the other three varieties, and large amounts of starch accumulation are observed (Figure 11). *B. oleracea* does not match the starch staining profile of the other *Brassicas* and in fact does not show significant starch accumulation throughout its life cycle (Figure 11). For the full sectional staining series see Appendix 3.


**Figure 11** Starch accumulation after a 5 week period in A – *B.rapa (pt)* B – *B.rapa (s)* C – *B.oleracea* by Lugol staining Presence of starch is indicated by black/blue staining within these sections.

### 3.2.2 *Raphanus sativus* Sections

*R.sativus* is within the same family (*Brassicaceae*) as the other *Brassicas* examined, and shares the same swollen hypocotyl structure. *R.sativus* was therefore also sectioned under the same time course to look for inter genus similarities that may be *LEC* derived. Growth was much faster than the other *Brassicas* and as a result only required 8 weeks of sections.

| | *A.* *R.sativus* Lugol stained | *B.* *R. sativus* Fat red stained |
|---|---|---|
| Week |   |   |
| Week |   |   |
| Week |   |   |
| Week |   |   |

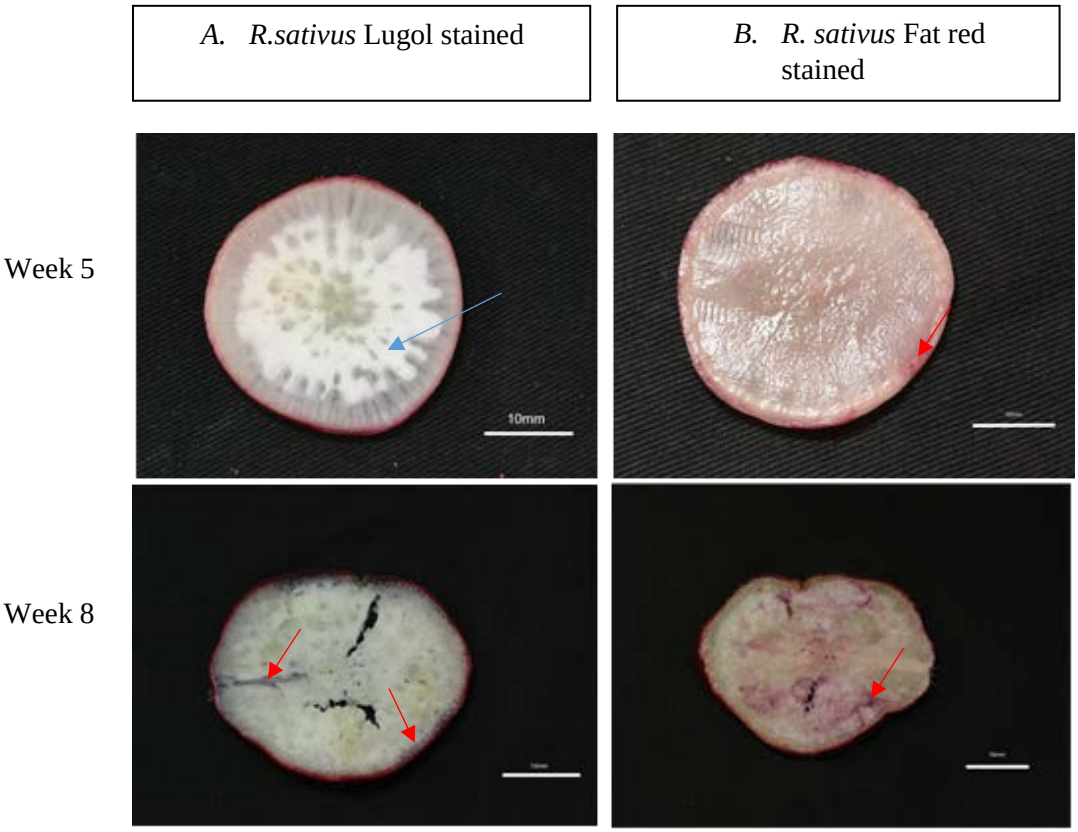



**Figure 12** Sections of *R.sativus* stained with Lugol for starch (A) and Sudan Red for lipids (B). The time course takes place over a 12 week period showing weeks 1-6, 8 and 12 where 12 represents a mature specimen. Week 1 represents 7 days after germination had been observed; subsequent weeks are also based from germination. Methods for sectioning and staining are explained in the methods sections. Red arrows indicate locations of starch/lipid presence; blue arrows highlight areas of particular absence.

Figure 12 shows the time course for *R.sativus* from one week after germination to maturity at week 8. Unlike *B.napus*, there is no population of starch granules located around the stele at week one (Figure 12, A), at this stage of growth the hypocotyl structure was already larger than its *Brassica* counterparts.

Starch accumulation appears to take place at an earlier point in *R.sativus* with the first positive stain occurring by week 2. This can be seen by the granules indicated within the xylem (Figure 12, A, Week 2). This accumulation pattern continues clearly in week 3 where significant hypocotyl swelling first occurs tripling in size (Figure 5, A, Week 3). At week 4, concentrated points of starch are observed in the outer xylem (See arrow, Figure 12, A, Week 5) but not the cambium, with starch granules thinly spread throughout. As the

organism grows, starch presence appears to lessen with a white pith like material taking up large portions of the hypocotyl (Figure 12, A, Week 5, blue arrow). By week 8 this tissue makes up the vast majority of the structure, where starch is only located around the very exterior of the xylem indicated by the red arrows.

Lipid staining is more similar to the pattern seen within the example species, *B.napus*. By week 1 there is already positive staining throughout, in a very similar manner seen in *B.napus* (Figure 12, B, Week 1). There appears to be a large accumulation at week 2 before hypocotyl expansion occurs where the whole structure appears to be saturated (Figure 12, B, Week 2). By week 3 the lipid location within the hypocotyl shares a similar pattern to that of the starch, with the exception that the cambium appears to contain significantly more lipids. This can be seen by the large amount of red stain indicated by the arrow (Figure 12, B, Week 3 - 4). Week 5 shows a decrease in overall storage compounds similar to that seen in the *Brassicas*, but the white pith when stained with Fat Red is much less obvious (Figure 12, B, Week 5-8)

### 3.2.3 Brassica napus var. napus – Oil seed rape

*B.napus var napus* was chosen as a control to examine whether hypocotyl starch presence was independent of hypocotyl swelling. Particular interest was placed in sections one week after germination to determine if starch seen within the *Brassicas* (Section 3.2.1) could be an embryonic carry over. The role of the hypocotyl in *B.napus napus* is of elongation post germination in order to find light and is later reinforced with lignin. This is due to the much larger nature of the stem compared to *Brassicas* we have examined. Most importantly, the hypocotyl does not swell and act as a storage organ so it is not expected to sequester starch.

Starch granules located around the stele are present in *B.napus var. napus* one week after germination as seen in the other *Brassica sp.* (Figure 13, A): The location of which is highlighted by the arrow. This is also mirrored in the lipids demonstrated by the Fat Red stain (Figure 13, B). The xylem hardens significantly throughout its life cycle and by week 5 there is still a small presence of starch in soft tissue between the exodermis and xylem

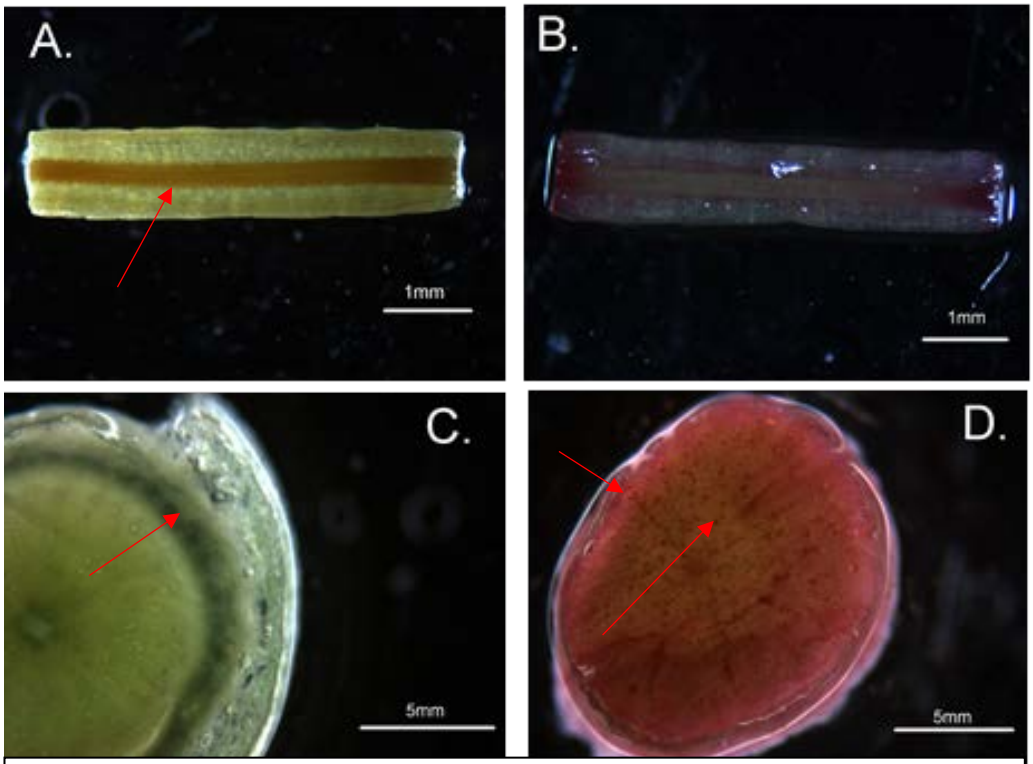



**Figure 13** Sectioned *Brassica napus var. napus.* A- Longitudinal section stained with Lugol, one week old. B - Longitudinal section stained with Fat Red, one week old. C – Section stained with Lugol, 5 weeks old. D – Section stained with fat red, 5 weeks old. Red arrows indicate locations of starch/lipid presence, blue highlight areas of particular absence.

(Figure 13, C), this is most likely a store for active transport and is indicated by the arrow. We see a similar profile in lipid storage located in the exterior circumference similar to starch Figure 13, D) with speckles of positive stain located throughout the xylem indicated within the figure.

### 3.2.4 Lignin staining

In older specimens, large sections of the hypocotyl stained negative for both starch and lipids. This was in stark contrast to the more uniform pattern seen in the younger sections. In order to determine the nature of the tissue, phloroglucinol was used to test for the presence of lignin as a structural component.

The unidentified white pithy material within the *R.sativus* hypocotyl did not stain red in the presence of phloroglucinol (Figure 14, A). Bright red staining was observed in the exodermis and at two points within the hypocotyl, these are indicated by the arrows in Figure 14, B. These areas were already naturally dark red due to the dye seen with the organism, but it is likely that there was some lignin content. Within *B.napus*, lignin was only observed in small quantities in the exodermis layer, the main component of the hypocotyl, the xylem, remained unstained Figure 14, D). Lignin was observed in small quantities in *B.rapa* var. snowball in populations throughout the xylem but in no other of the subject *Brassica* species. *B.napus var. napus* showed strongest lignin indication in which the entire

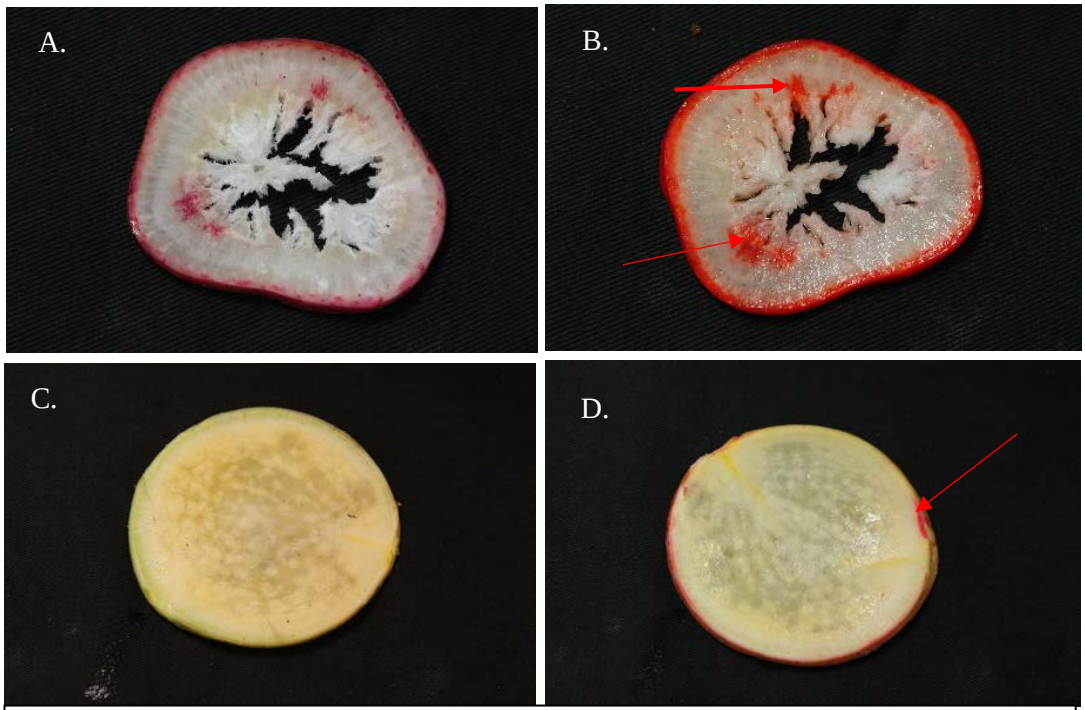



**Figure 14** Lignin staining using Phloroglucinol, samples 8 weeks old. A – *R.sativus*, unstained. B - *R.sativus*, stained. C – *B.napus* unstained. C – *B.napus* stained. Red staining indicates the presence of lignin highlighted by arrows.

xylem was uniformly stained. This was indicative of the structural properties observed Figure 14, C). It is worth noting that in *Brassicas* the later part of its life cycle involves the growth of a large stem on which the flowers are grown. It is possible the lignin seen here is in preparation to support this stem.

### 3.3 Embryonic screens

To further confirm the starch granules observed in the week one *Brassica sp.* sections (Figure 9, A) were a carry-over from embryonic development, as opposed to active accumulation in the post germination hypocotyl, pre germination hypocotyls were examined for similar patterning. In *Arabidopsis*, it is known most of the storage compounds in the seed are located in the cotyledons and hypocotyl (Maheshwarl, 1950) but not the extent to which they persist in the germinated tissue. In *Brassicas* the seeds are much larger and store their compounds externally to the embryo.

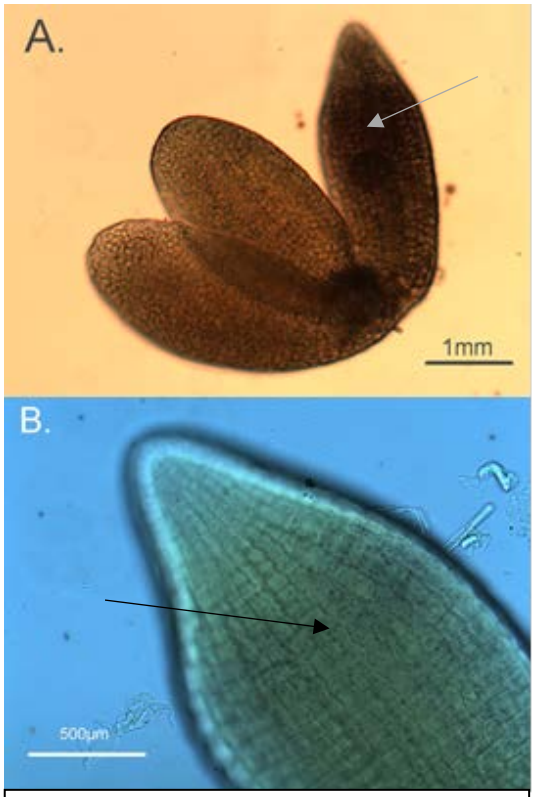



**Figure 15** *Arabidopsis* embryos stained for starch using Lugol and cleared using Histoclear. A – Whole embryo showing staining throughout the organism particularly the root-hypocotyl junction. B – Individual starch grains in the root hypocotyl junction.

Wildtype *Arabidopsis* embryos stained positive for the presence of starch in the hypocotyl in a similar manner seen in the *Brassicas sp.* (Figure 15.) Similar patterns were observed in both *Col 0* and the *tnp* mutant, so it did not appear to be a phenotype indicative of the *tnp* mutant and elevated levels of *LEC1*expression. The large presence of starch in the hypocotyl of the embryo is indicated in Figure 15, A whilst the individual starch granules can be seen within Figure 15, B at the higher magnification.

*Brassica* embryos could not be examined as soft seeds were not obtainable at the time; the embryos were consistently destroyed in attempts to remove them from dried seeds the plants were grown from.

The presence of the starch observed here could correlate to the starch seen in the wildtype *Arabidopsis* grown under 2, 4 – D as an artefact and may be of embryonic origin.

Alternatively, it could suggest the starch present may be a result of inhibition of the starch breakdown caused by the increased levels of auxin.

### 3.4 *Brassica* gene expression

#### 3.4.1 *LEC1* expression in a *Brassica* time course within the hypocotyl and leaf

In order to determine if *LECs* were present in vegetative tissue, which previous experiments have suggested (Tilley E., *Unpublished data University of Durham*), RNA was extracted from each of the same time points in which the *Brassicas* were sectioned in Section 3.2. The RNA extraction was to determine quantative expression levels at discreet points which previously had not been demonstrated. The expression levels were then compared to the stained sections in order to determine if there was a correlation between starch and lipid accumulation and the *LEC* expression levels.

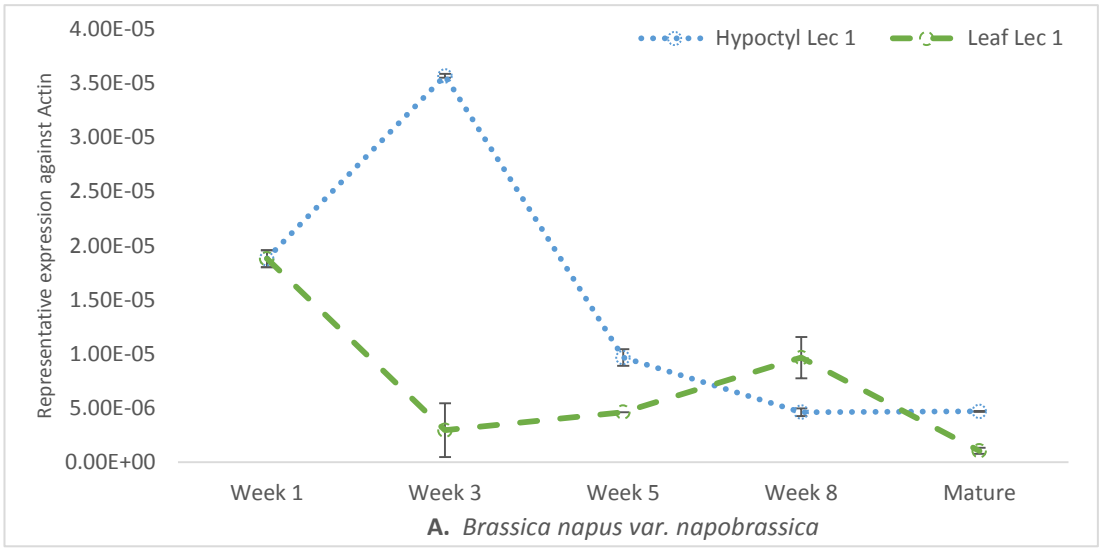
The relative expression levels for *LEC1* across all 5 of the species were found to be similar. Generally higher values were observed within the two *B.rapa* varieties (Figure 16). Not only the values but the trend during development showed similar characterisitics, starting somewhat variably but increasing noticeably at week 3 and becoming lower as the organisms matured (Figure 16).

The appropriate statistical analysis for data of this form would ideally be either a polynomial reggression or a non independent ANOVA using a post hoc Tukey test. This would acknowledge the sections highlighted by the time course and the ANOVA would not make any assumptions about the shape curve which is clearly not linear (Figure 16). As an initial analysis a T-test with Bonferroni correction for multiple comparisons was used, which although giving a higher risk of false negatives, highlights the periods of extreme change which are visulised in Figure 16. Due to the nature of the multiple comparison T test, a significance value was calculated to be <0.02.

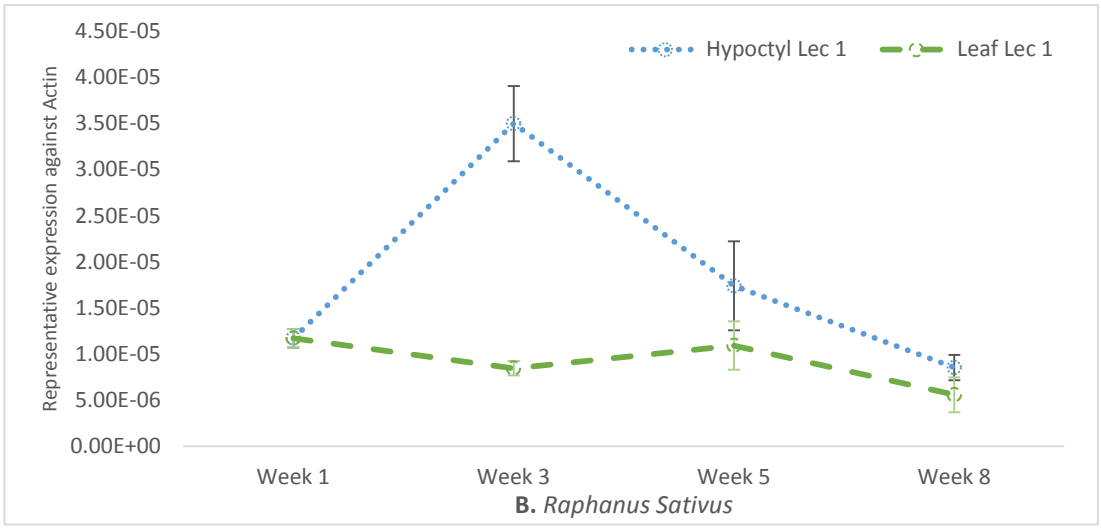
The highest level of *LEC1* expression across the species is at week 3 in the hypocotyl, ie shortly before hypocotyl expansion and starch accumulation was observed (Figure 9, A-3,

Week 3 peak). Using week 1 as a baseline and comparing week 3, the results are now decribed for *B.napus, R.sativus, B.oleracea, B.rapa (pt)* and *B.rapa(s)* (students t-test, $P<0.001$ df = 4, $P = 0.023$ df = 2, $P =0.087$ df =3, $P= 0.015$ df = 4, $P<0.001$ df =3 respectivley) and to avoid repetition, the same reporting order shall be used unless stated otherwise.

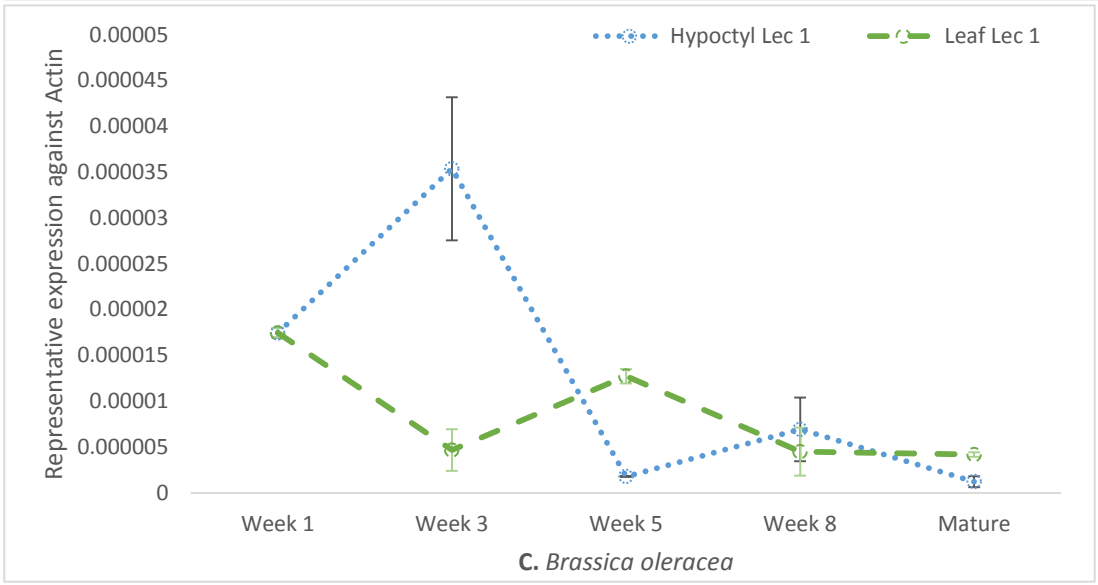
At most points *LEC1* expression was higher in hypocotyl tissue compared to leaf and was significantly so at week 3 in most instances (Students t-test, $P=0.007$ df =3, $P=0.018$ df = 2, $P = 0.029$ df = 3, $P = 0.007$ df = 4, $P<0.001$ df =4). By the time the *Brassicas* were mature, expression levels had dropped off but to a variable level, whereby in some instances the values were similar to the week 1 baseline, in other cases it was significantly lower. In all cases the levels were signifcantly lower than the week 3 spike ( $P = 0.002$ df =3, $P = 0.04$ df =3, $P = 0.02$ df = 3, $P = 0.02$ df = 3, $P>0.001$ df = 4).



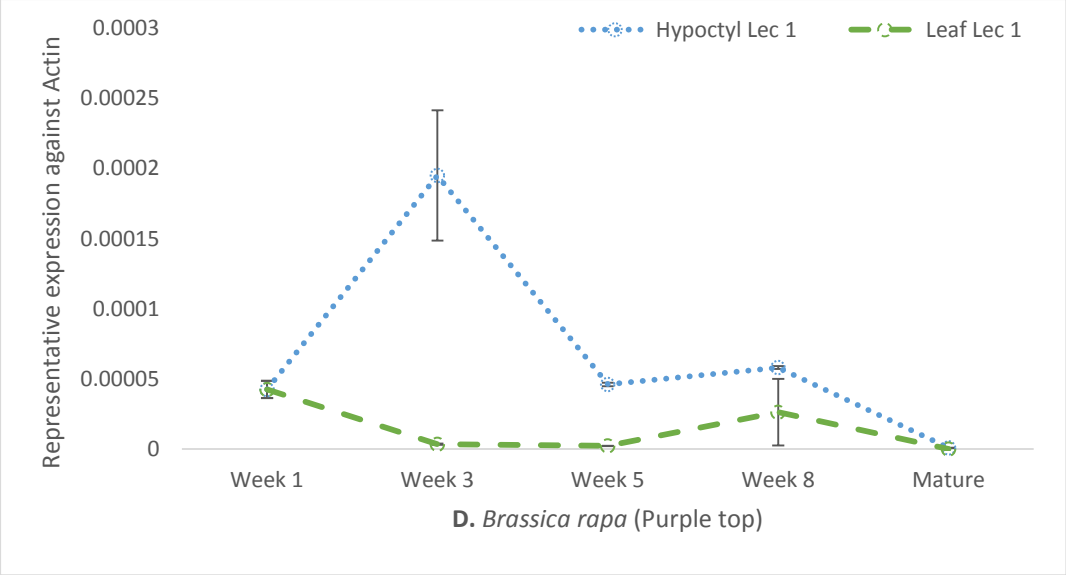

**A.** *Brassica napus var. napobrassica*




**B.** *Raphanus Sativus*




**C.** *Brassica oleracea*



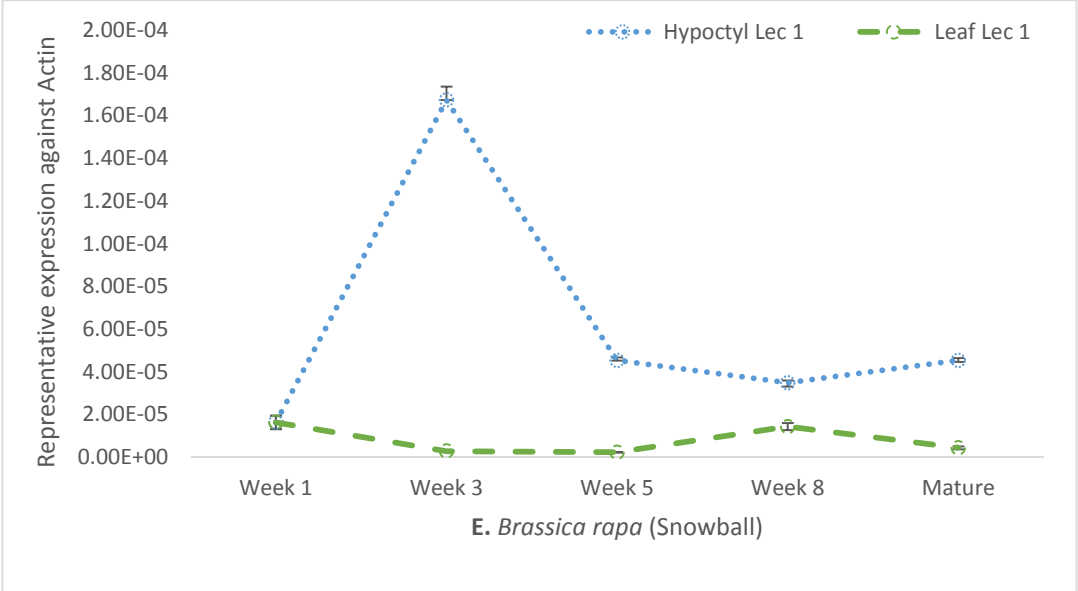




**Figure 16** Relative expression levels of *LEC1* (Against the house keeping gene Actin) in A - *B.napus* B – *R.sativus* C – *B.oleracea* D – *B.rapa pt.* E – *B.rapa s.* 'n' weeks after germination where M represents a mature specimen 12 weeks after germination. Values calculated from three technical repeats. Error bars represent +/- SE

### 3.4.2 *LEC2* expression in a *Brassica* time course within the hypocotyl and leaf

The expression profiles for *LEC2* across all 5 of the species once again are similar, with the exception of *B.oleracea*. The overall relative expression levels were highest in the two varieties of *B. rapa* and in *B. napus* (Figure 18). The trend seems in direct contrast to *LEC1* (Figure 16, Week 3), starting off low and after week 3 increasing significantly (Figure 16, A,C-E). The highest level of *LEC2* expression was around week 5 after hypcotyl expansion has begun to occur and starch accumulation begins (Figure 18, C-E). Week 3 was used as a comparison point for *B.napus, R.sativus, B.oleracea, B.rapa (pt)* and *B.rapa(s)* respectively ($P>0.001$ df = 3, $P = 0.02$ df = 3, $P = 0.36$ df = 3, $P = 0.05$ df = 3, $P = 0.02$ df = 3). *B.napus*, was the only sample in which LEC2 expression levels at week 8 were higher than at week 5 (two sample t-test, $P<0.001$, df = 3) . At most developmental stages *LEC2* expression was higher in hypocotyl tissue compared to leaf and was significant in all cases at week 5 (Students t-test, $P <0.001$ df = 2, $P<0.001$ df = 3, $P = 0.042$ df = 3, $P = 0.006$ df = 3, $P< 0.001$ df = 3).

*B.oleracea* was different in that there was no week 5 increase in *LEC2* expression. The initial peak is comparible to the other *Brassicas,* but transcipt levels decrease throughout the rest of the life cycle . This was correlated with the apparent lack of starch accumulation seen in the *B.oleracea* sectional series (Figure 17). For entire staining series see the appendix 6.

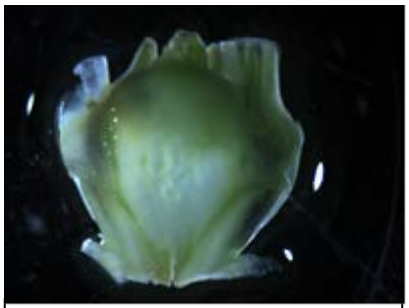
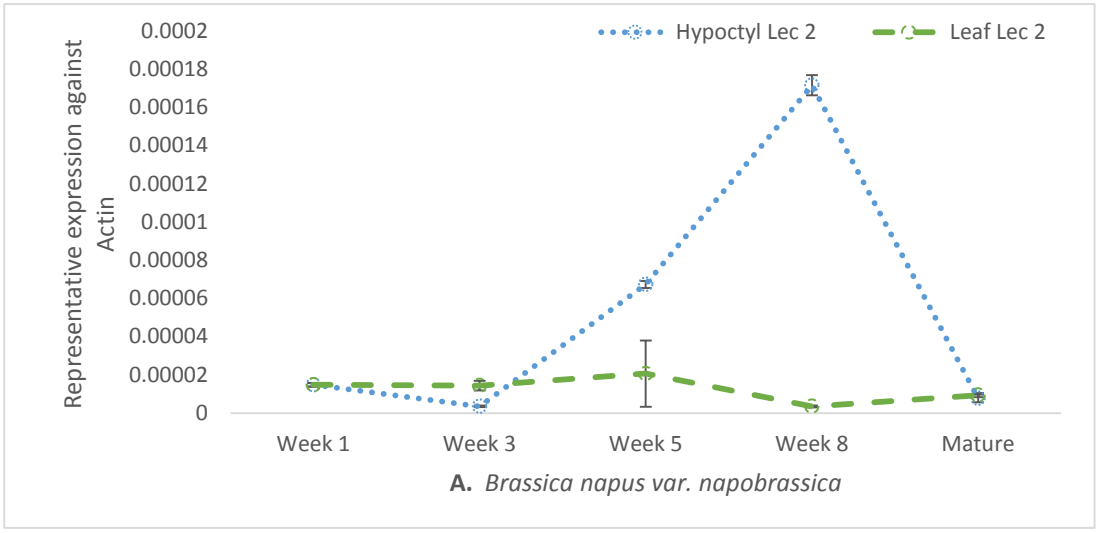
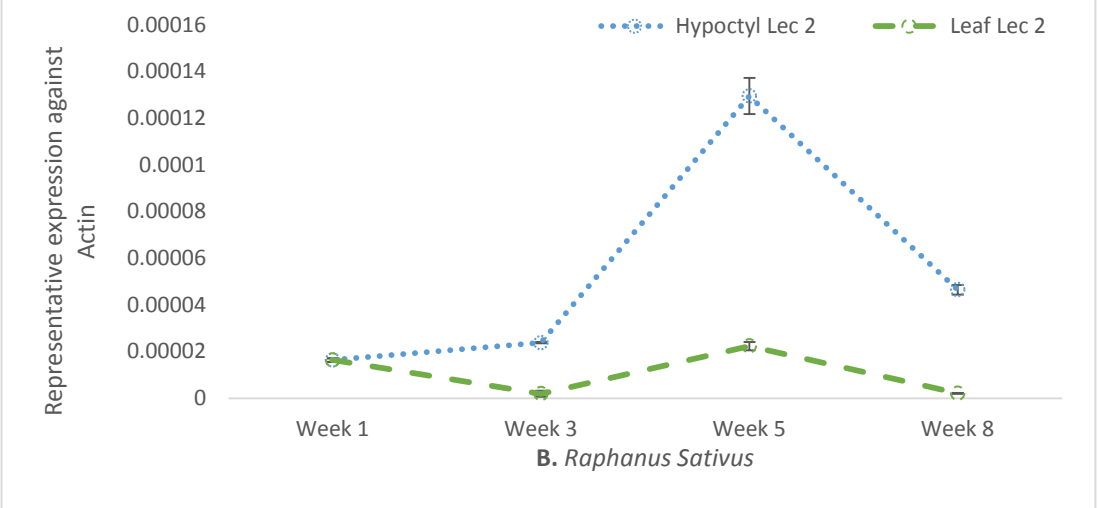


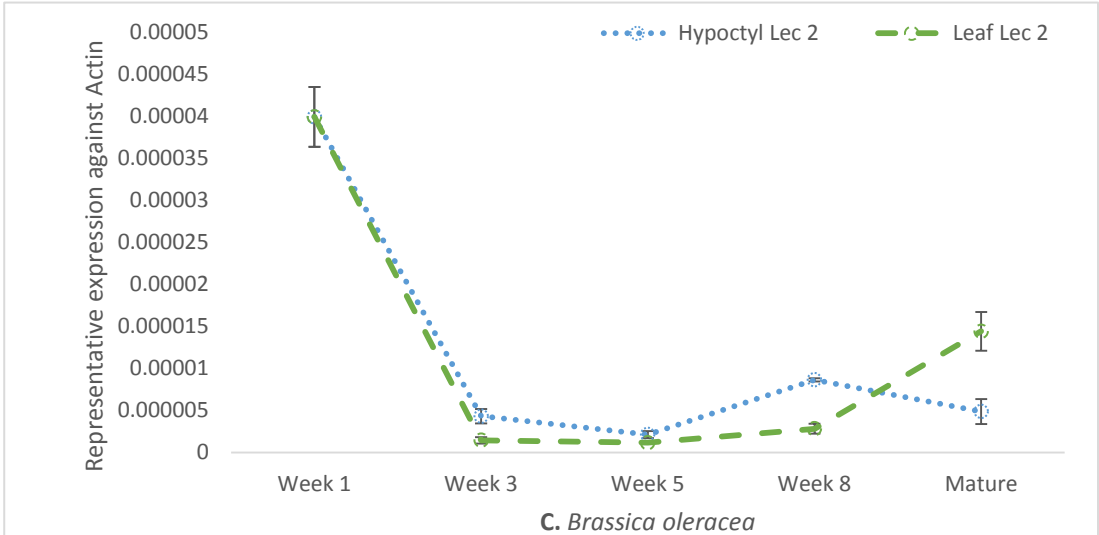
**Figure 17** 5 week old *B.oleracae* stained with Lugol for starch showing significantly less staining than the other *Brassicas* at the same time point.



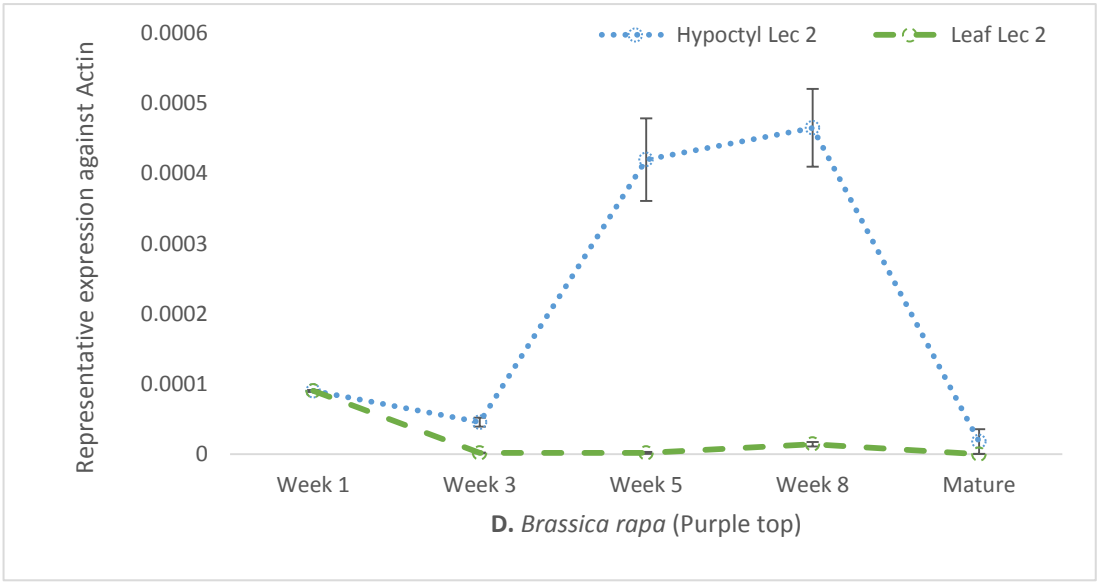

**A.** *Brassica napus var. napobrassica*




**B.** *Raphanus Sativus*




**C.** *Brassica oleracea*



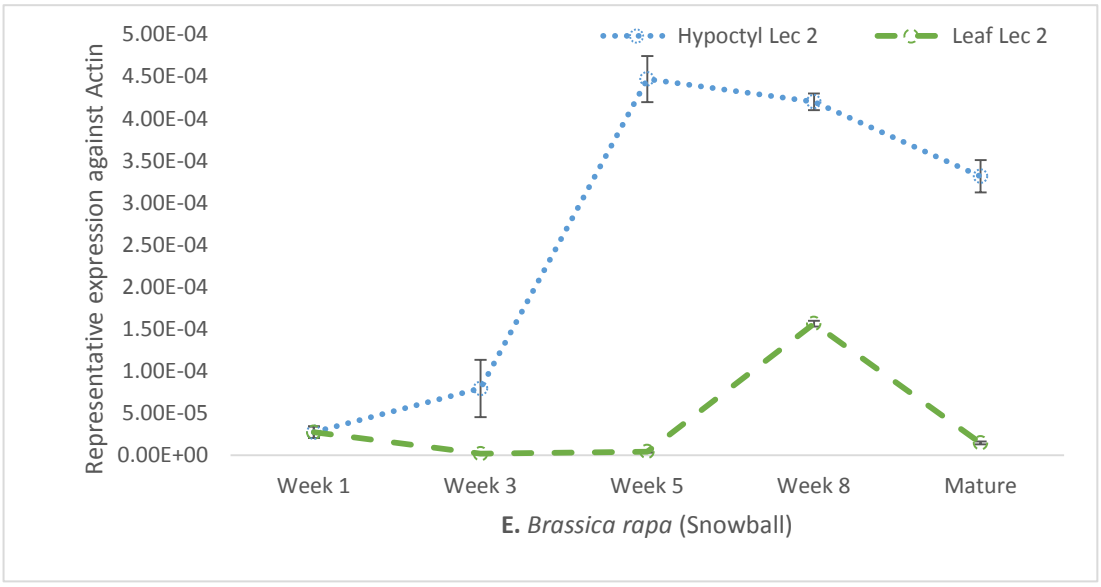




**Figure 18** Relative expression levels of *LEC2* (Against the house keeping gene Actin) in A - *B.napus* B – *R.sativus* C – *B.oleracea* D – *B.rapa pt.* E – *B.rapa s.* 'n' weeks after germination where M represents a mature specimen 12 weeks after germination. Values calculated from three technical repeats. Error bars represent +/- SE

### 3.4.3 *FUS3* expression in a *Brassica* time course within the hypocotyl and leaf

There were plans to investigate *FUS3* expression as it is a B3 binding domain transcription factor similar to LEC2, however, this was not possible due to non-specific primers and insufficient time to redesign new ones.

### 3.5 *Arabidopsis* seed composition

Previous studies of the *tnp* mutant revealed no adverse effects of the mutation on embryo formation (Casson & Lindsey, 2006). It was therefore presumed that there was no change in *LEC1* expression in embryogenesis, and therefore no embryonic phenotype. Although there was no obvious patterning or proportional body plan change, it is possible that there could be an effect on the quantity of storage compounds laid down before dormancy.

To investigate whether the unregulated expression of *LEC1* affected the size of *Arabidopsis* seeds, the width and estimated volume of the *tnp* seeds were compared to the Col-0 equivalent (Figure 19). Volume was calculated as $V = 4/3 \times \pi \times L \times W^2$ where L is length and W is width in mm.

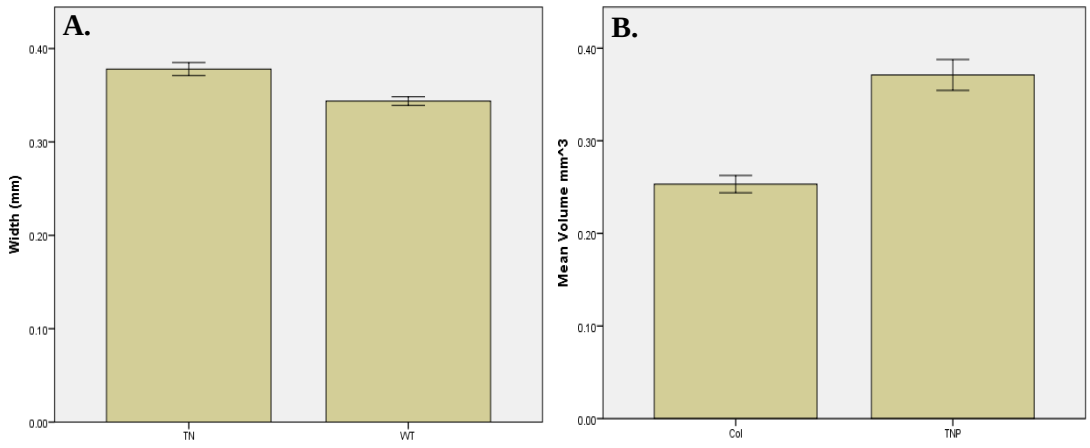



**Figure 19** Comparison of *Arabidopsis thaliana* seeds between the *tnp* mutant and *Col 0* wildtype. (A) Width Comparison (B) Estimated volume comparison using width and length of seeds. All error bars show mean +/- 2 SE

In cases of both width and volume the *tnp* seeds were larger than their *Col 0* counterpart. The volume comparison showed the largest mean difference of $0.118mm^3$ (Independent variable t-test, $p < 0.001$, d.f. = 179), but a width comparison at the largest point was equally able to show a significant mean difference of 340μm (Independent variable t-test, $p < 0.001$, d.f. = 180). This suggests that there is a *tnp* phenotype in the seed structure of embryo size, if not in the patterning of the embryo.

### 3.6 Fatty acid comparison

It was hypothesised that the observed increase in seed size was due to an increase in the storage compounds present. Due to the absence of the promotor sequence in the 35S::LEC1 transgenic, *LEC1* expression would be unregulated, allowing a larger build-up of starch and lipids. This could cause the seeds to be larger. Total fatty acid analysis through Gas Chromatography was therefore carried out to determine if there was indeed a significant increase in some of these storage compounds.

#### 3.6.1 *Arabidopsis* analysis

First of all we had to determine whether the method was extracting a consistent and reasonable level of fatty acids. Seeds are generally ~40% fatty acid content by weight (O'Neil et al., 2003), and it was determined our average seed weight was 25 ng (n = 100 seeds). Each fatty acid extraction yielded 12.9 ng (+/- 0.4 SD) per seed, which constitutes ~50% of the seed by mass, and it was therefore concluded that the method is suitable for extracting fatty acids reliably. The fatty acid profile is shown in Figure 20.

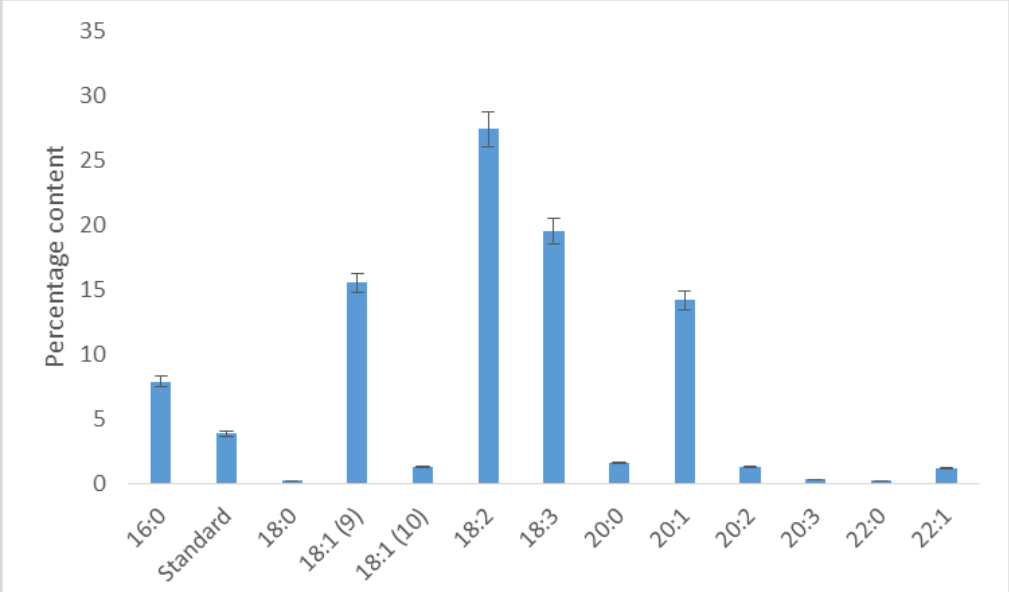



**Figure 20** Fatty acid percentage profile of *Arabidopsis* wildtype seeds from 20 samples, where fatty acids are represented by their carbon chain length and number of double bonds. Ratios calculated from known standard of carbon chain 17. Error bars represent +/- 2SE.

**Table 11. Fatty acid composition of *Arabidopsis seeds* vs published data. Data are means expressed as amount of fatty acid (ng) per seed. This was in order to illustrate the method of extraction was working correctly. Fatty acids are represented by their carbon chain length and double bond number.**

| Fatty Acids | Weight per seed (ng) | Y.H.Li et al., 2006 |
|---|---|---|
| 16:0 | 1.0 | 1.5 |
| 18:0 | 0.0 | 0.2 |
| 18:1 (9) | 2.0 | 2.2 |
| 18:1 (10) | 0.2 | - |
| 18:2 | 3.5 | 4.8 |
| 18:3 | 2.5 | 3.2 |
| 20:0 | 0.2 | 0.4 |
| 20:1 | 1.8 | 3.4 |
| 20:2 | 0.2 | 0.3 |
| 20:3 | 0.0 | - |
| 22:0 | 0.0 | - |
| 22:1 | 0.2 | 0.3 |

A comparison of fatty acids extracted shows the data observed are consistent with previously published data (Table 11), allowing valid comparisons to be made between the mutants and storage tissues. Different methods for extraction were tried including crushing the seeds beforehand. The highest yielding method was using whole seeds as opposed to crushed, which resulted in a 40% yield drop. This is most likely due to an inability to remove all material from the mortar and pestle.

When the fatty acid profile of the wildtype seed is compared to that of *tnp* mutant (Figure 21) a significantly higher level in three of the peaks was found:, 18:2, 18:3 and 20:1 in the mutant (Independent Samples t test, P = 0.045, df = 2, p 0.041 df = 3, p =0.036 df = 3). The original mean volume difference of 0.18mm$^3$ can be described as a 1.46x increase in overall volume. The increased fatty acid weight of *tnp* vs *Col 0* was a 2.02ng (+/- 0.1) increase

correlating to 1.37x (+/- 0.2) difference. This supports the view that the observed seed volume increase is associated with an increased fatty acid accumulation in the seed.

In the wildtype *Arabidopsis* seedlings a very similar profile increase is observed in the *tnp* mutant. The mutant seedlings show an overall increase in the fatty acids 18:2, 18:3 and 20:1 1 (Independent Samples t test, P = 0.025, df = 4, p 0.051 df = 3, p =0.046 df = 3) compared to wildtype.

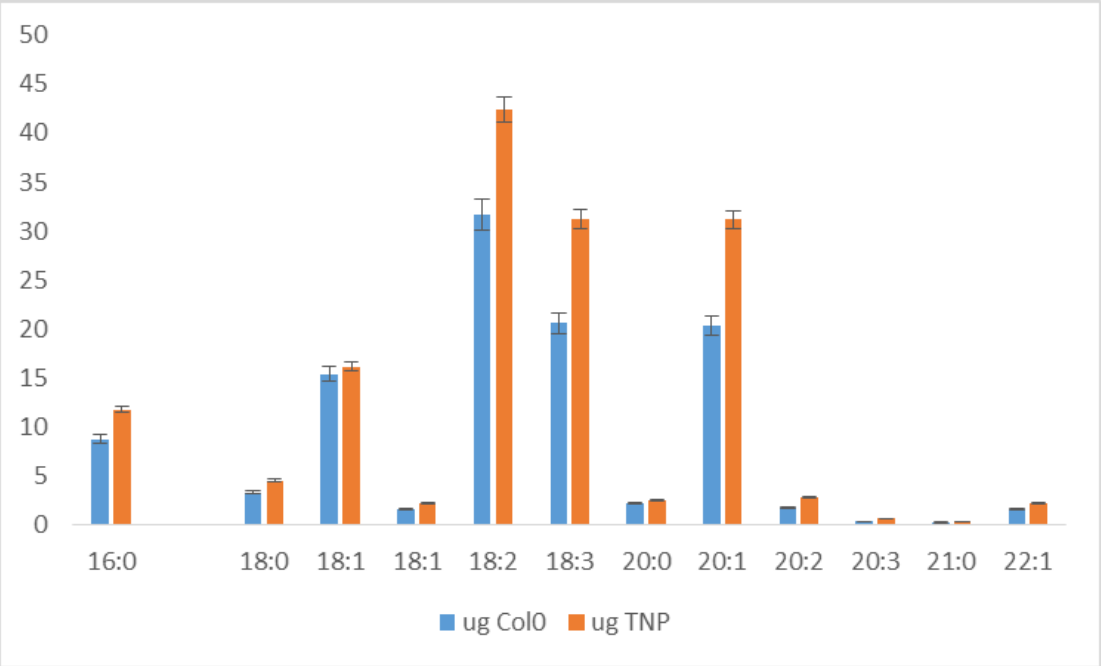



**Figure 21** Fatty acid profile comparison *Arabidopsis Col0 vs tnp*, 20 seeds used for each reaction. Fatty acids displayed as carbon chain length and double bond number. Error bars represent +/- 2SE.

### 3.6.2 *Brassicaceae* seed analysis

*Brassicaceae* seeds vary in morphology between species and varieties (Table 12) where the smallest seed (*Arabidopsis)* weighs 334 times less than the largest (*R.sativus).* Average weights were calculated from 100 seeds. In order to compare between species, percentage fatty acid composition was used.

**Table 12. Seed weights of *Brassicas* calculated from 100 seeds. +/- represents Standard deviation**

| **Species** | **Weight per seed (µg)** |
|---|---|
| *Arabidopsis thaliana* | 0.025 (+/- 0.05) |
| *Raphanus sativus* | 8.35(+/- 0.8) |
| *Brassica rapa (pt)* | 1.89(+/- 0.1) |
| *Brassica rapa (s)* | 1.85(+/- 0.1) |
| *Brassica oleracea* | 4.30(+/- 0.2) |
| *Brassica napus* | 2.14(+/- 0.1 |

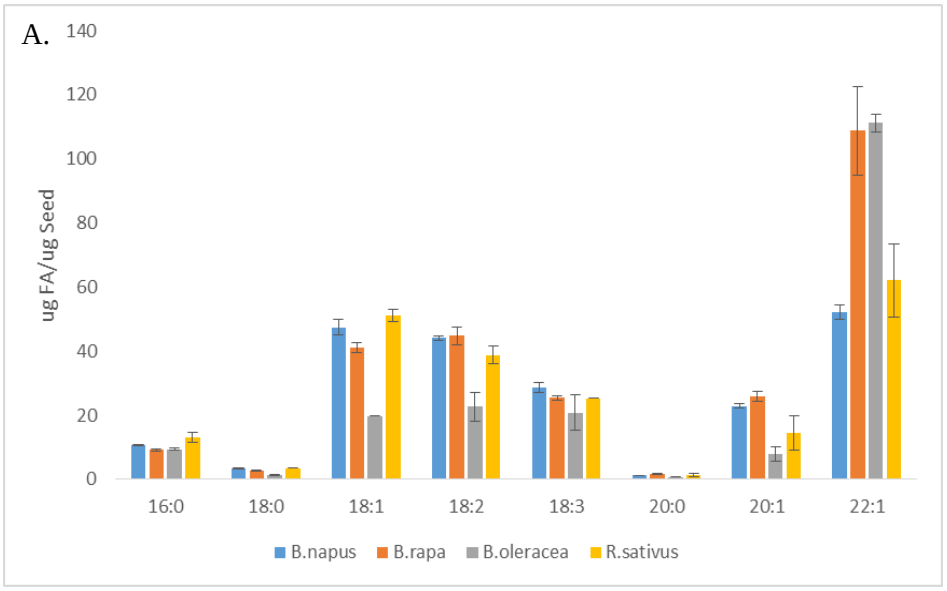
*Brassica* seeds were found to show a similar fatty acid percentage composition pattern. There were relatively high amounts of the fatty acids 18:1, 18:2, 18:3 and 22:1, the later which is most likely to be erucic acid (Figure 22, A).

The ideal way to analyse this data would be an ordinance technique such as CCA (Categorical Component Analysis) followed by a GLM (Generalised Linear Model) test for redundancy using an AIC (Akaike Information Criterion). However, as a preliminary analysis, I have decided to use bi-plots to approximate the ordination method (Figure 22).

*B.oleracae* has significantly less FA per seed in two of the most abundant fatty acids, 18:1 and 18:2 (One way anova, $P<0.001$, df = 3, Tukey Test, 14.2, 5.9, 22.4, $P<0.05$) (One way anova, $P = 0.003$, Tukey Test, 8.9, 5.1, 5.7 $P<0.05$). For 18:3 there was found to be no significant differences between any of the samples (One way anova, $p = 0.3689$). In *B.napus, B.rapa* & *R.sativus* the values for 18:1 and 18:2 were very similar (Figure 22, A).

As the fatty acid 22:1 was not present within *Arabidopsis* (Figure 21) these peaks were omitted for the comparison between the other *Brassicas* and *Arabidopsis* (Figure 21, B)(Figure 22). Percentage composition was used to allow comparison with the significantly smaller *Arabidopsis* seed (25 ng) and therefore fatty acid content. For 18:1 all of the *Brassicas* except *B.oleracea* showed a significantly higher level than *B.napus, R.sativus, B.oleracea, B.rapa (pt)* and *B.rapa(s)* respectively (Figure 23, B) (One way anova, $P<0.001$,

Tukey test, Q = 14.68 P<0.01, Q = 15.16 P<0.01 Q = 0.68 P = 0.89, Q= 6.49 P = 0.01). For the fatty acid 18:2 the percentage composition values were very similar between all samples (Figure 23, D)(One way anova, df =4 P = 0.53) as were those for 18:3 except between *tnp* and *B.rapa* (Figure 23, E) (Tukey test, Q = 10.6, P = 0.001).



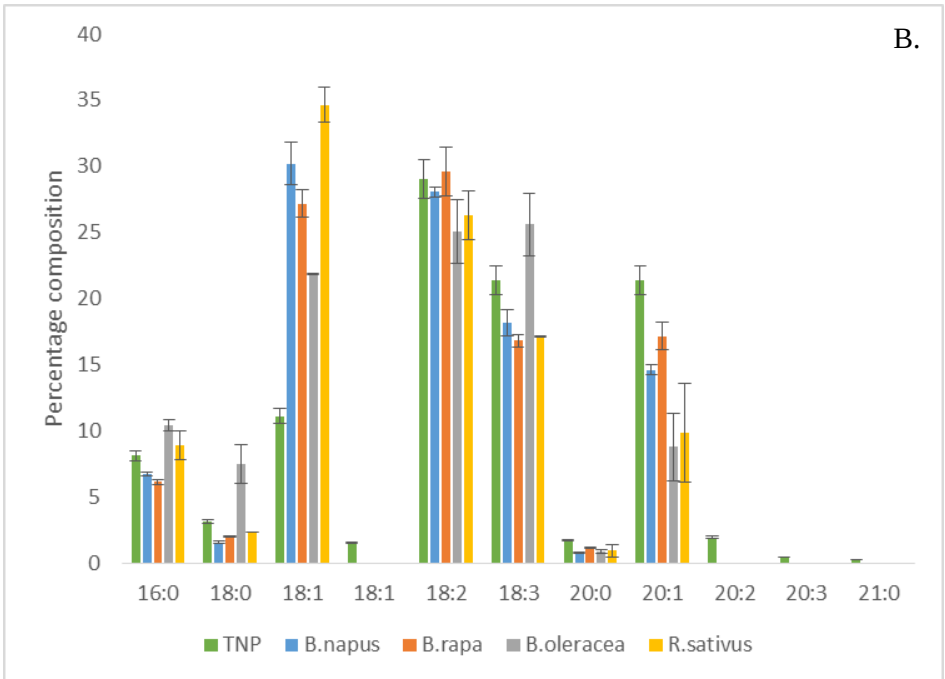




**Figure 22** Fatty acid composition of *Brassicas*, 2 seeds used for each reaction. A – *Brassica* complete profile. B – *Brassica* & *Arabidopsis* without Fatty acid 22:1. Values calculated from 3 biological repeats Error bars represent +/- 2SE.

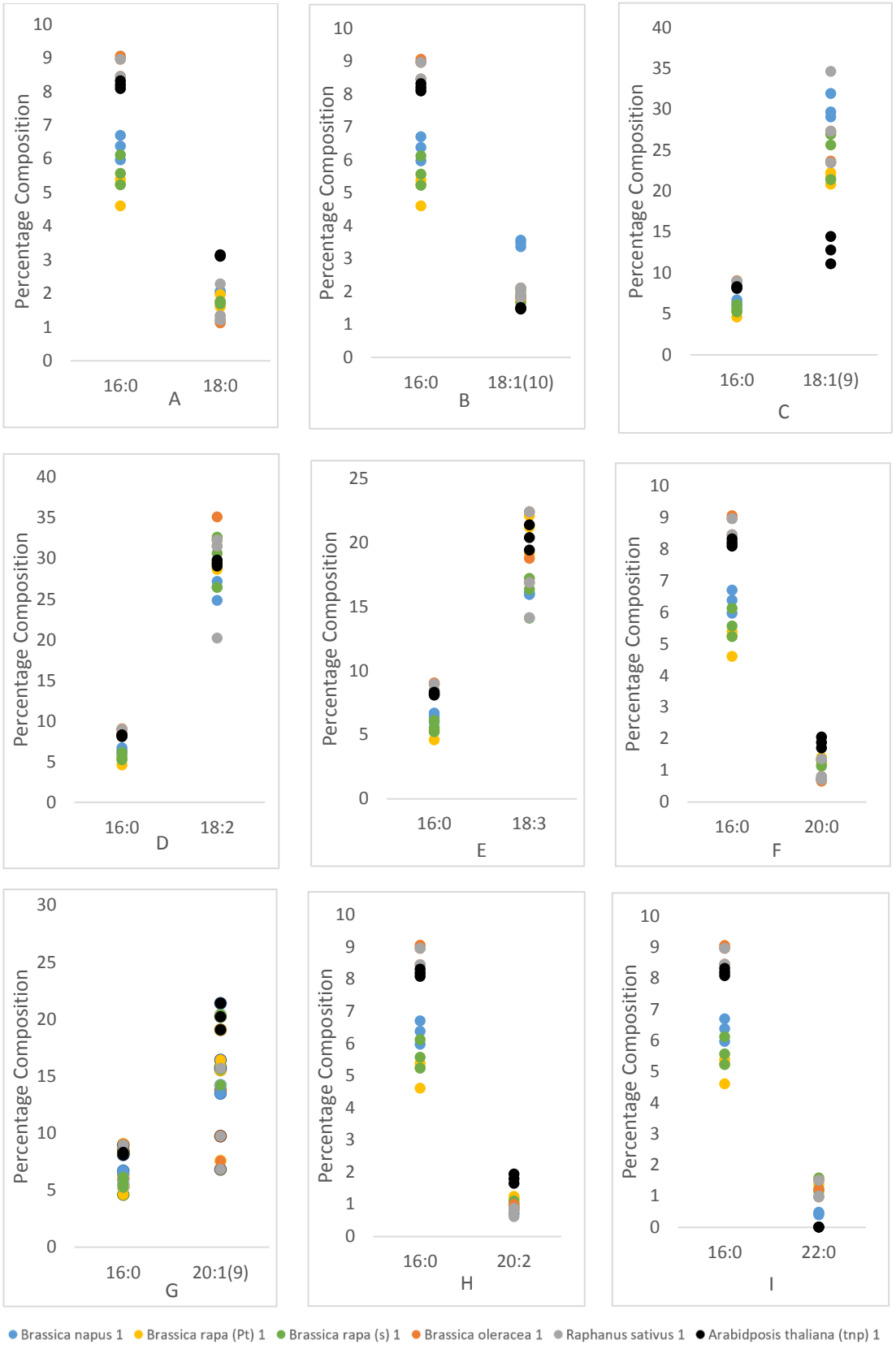



**Figure 23** Fatty acid percentage composition of *Brassica* seeds presented as a bi plot. Each fatty acid is compared against 16:0 to highlight the spread between species and highlight higher or lower compositions that were less obvious in **Figure 21**. Two seeds used for each reaction, each species represented three times.

### 3.6.3 *Brassica* tissue analysis

Samples from adult hypocotyls (8 weeks) were analysed to determine whether the fatty acids present were similar to those observed in the *tnp* mutant (3.6.3). The fatty acids that were demonstrated to increase in the mutant vs the wildtype, 18:2, 18:3 and 21:0 were of particular interest.

Initially *B.napus* hypocotyl and leaf were compared to determine which fatty acids showed a significant increase in the hypocotyl (Figure 24). In all cases except the fatty acid chains 20:0 & 22:1, the hypocotyl contained increased levels of all the major fatty acids present. At the point of greatest similarity (Students t-test, df = 4 P =0.003).

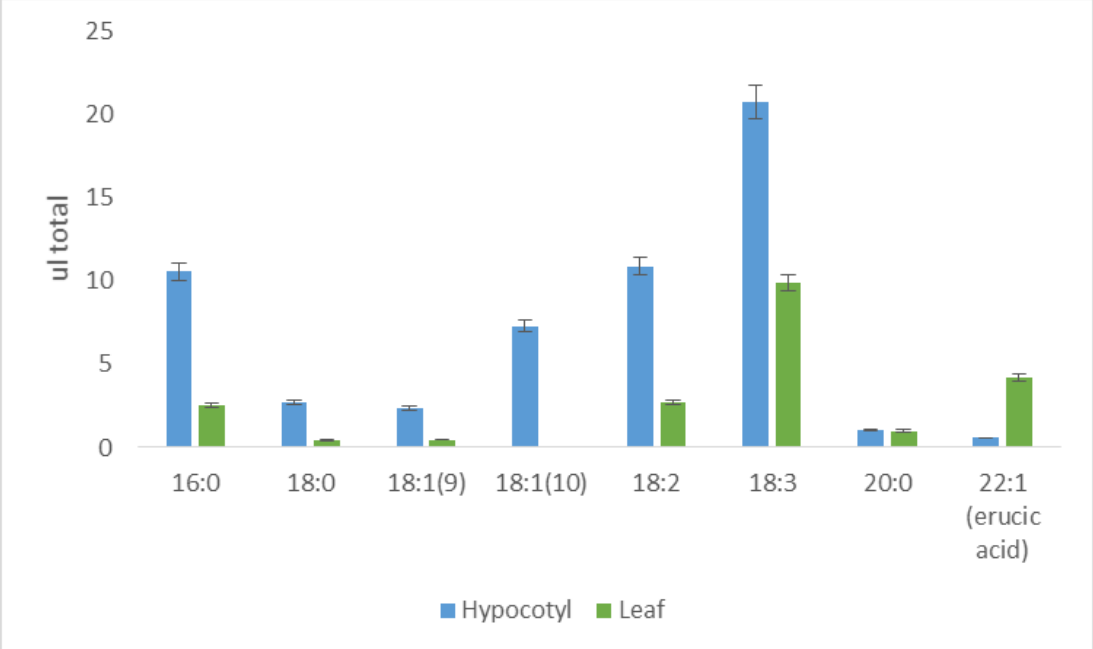



**Figure 24** Fatty acid composition of *Brassica* tissue, 1g used for each reaction, 100mg used for GC run. A – *Brassica napus hypocotyl vs Brassica napus leaf*. B – *B.napus & B.rapa* profiles. Values calculated from 3 biological repeats Error bars represent +/- 2SE.

Comparisons between *Brassicas* were then carried out with the exception of *B.rapa*, for which it was not possible to produce consistent values; and *R.sativus* mature tissue was not available for analysis. The fatty acid profiles between *B.napus* and *B.oleracea* showed very similar values with major peaks at 16:0, 18:2 and 18:3 (Figure 25, C). The large relative amounts of 18:2 and 18:3 are of particular interest as these were two of the fatty acids present in high levels within the *tnp* mutant (Figure 21). a significantly higher abundance of 18:3 was found in the hypocotyl compared with the seed in both *B.napus* and *B.oleracea*. (Two samples T-Test, df = 3, $P<0.001$, df =4 $P<0.007$ respectively) In contrast the fatty acid 18:2 showed no difference in relative abundance between the samples (Two samples T-Test, df = 3, P=0.95, df =4 P=0.58).

Using the seed as a baseline for comparison, the adult *B.oleracea* and *B.napus* hypocotyl fatty acid contents were compared. The two fatty acids of interest, 18:2 and 18:3 showed interesting changes from the seed to the hypocotyl even when the fatty 22:1 is removed from the percentage composition. This was removed as it constituted 50% of the seed content in *B.oleracea* and therefore skewed the other results negatively. In the seed we would expect to find fatty acids in high concentration for use in storage and desiccation protection, so a 50% change therefore represents a relatively high concentration in the hypocotyl. In the case of the fatty acid 18:2 there is only a 7% and 12% decrease in *B.napus* and *B.oleracea* respectively. For fatty acid 18:3 a 22% and 20% increase from seed levels are observed.

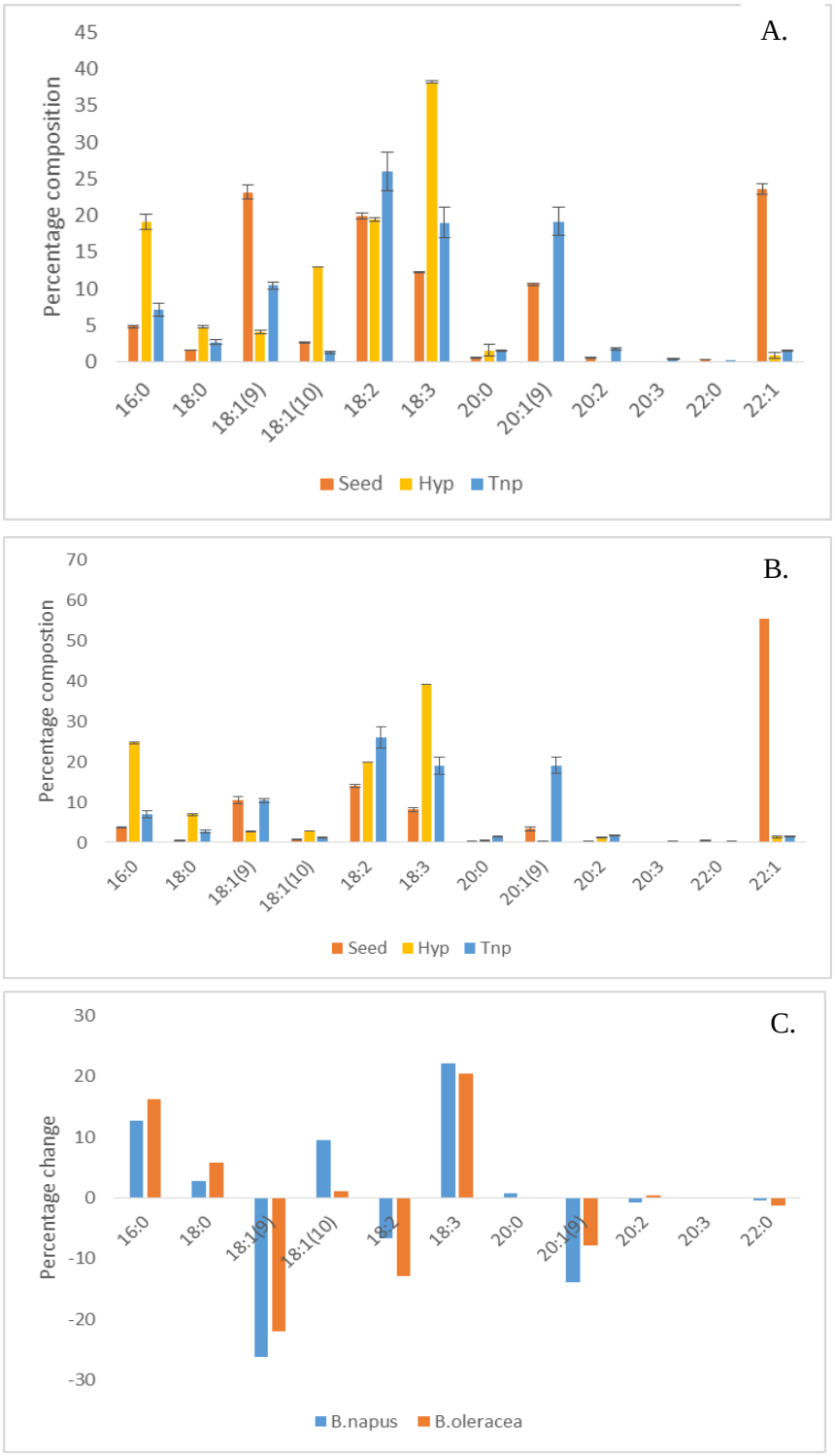



**Figure 25**. Fatty acid composition of *Brassicas* tissues, A – *Brassica napus*. B –*Brassica oleracea*. C – Percentage change in Fatty acid composition between seed and adult hypocotyl. Values calculated from 3 biological repeats Error bars represent +/- 2SE.

The adult hypocotyl was compared to a 6 day old seedling in *B.oleracea* (Figure 26). The results of which are somewhat surprising, as the hypocotyl contained less fatty acids per µg of WT tissue than the seedlings of the same species. For 18:2 an 18:3, the seedling contained 53 µg/g and 97 µg/g more respectively (T-Test, P=0.03, P>0.01). Due to time restraints of optimisation we were only able to compare *B.oleracea* seedlings vs. hypocotyl.

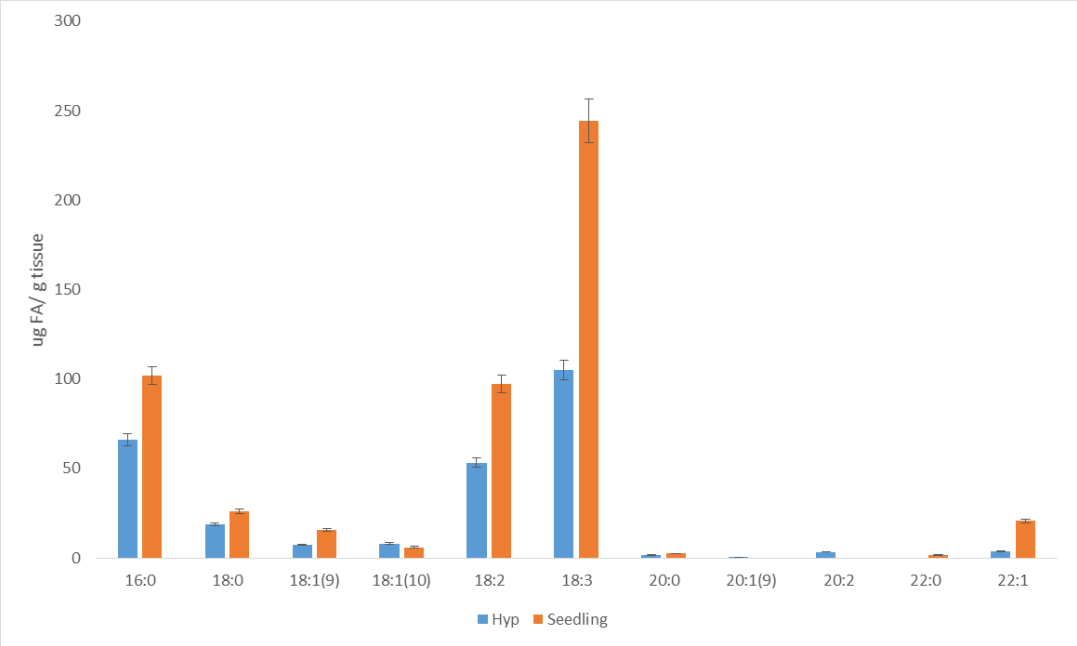



**Figure 26** Fatty acid composition of *Brassica oleracea* 6 day old seedling vs 8 week hypocotyl. Error bars represent +/- 2 SE.

# 4 Discussion

The role of *LEC* genes as regulators of embryogenesis is well documented in the literature (West et al., 1994; Gaj et al., 2005; Braybrook and Harada, 2008). Expression of *LECs* is almost exclusively shown to take place before germination, playing roles in embryonic tissue identity and storage product accumulation. There is evidence of repression through the promotor region in vegetative tissue via *PKL,* and evidence of cross talk between LEC1, LEC2, FUS3, ABI3 and hormones such as auxin and gibberellins. Gibberellins are largely involved in the transition of the seed from dormancy to germination and repression of *LEC* genes (Ogas et al., 1997).

Ectopic expression is rarely observed, and of particular interest are the findings by Casson and Lindsey (2006) who reported *LEC1* expression in *Arabidopsis* seedlings, due to a gain-of-function mutation in the *LEC1* gene. The result of this was embryonic-like cells present in hypocotyl which contained high levels of starch and storage lipids, morphologically similar to a turnip and hence the name, the *turnip* mutant (*tnp*). The high levels of oil present within various *Brassicas* (Ahuja et al., 1987) and the activation of morphogenetic pathways normally only active in the embryo producing dense storage tissue within vegetative organs promoted questions about the evolution of the brassica storage organs and expression of *LECs*. Were *LECs* responsible for the same vegetative structures in *Brassicas* and if so, why is the only phenotype observed in the hypocotyl?

## 4.1 *LEC1* and the *tnp* mutant

When the *tnp* mutant is grown on media containing the synthetic auxin 2, 4-D and 30g/L of sucrose, the incidence of penetrance is increased. When the mutant phenotype is evident, altered cell identity is observed at the swollen junction between the hypocotyl and the root (Figure 7) and stains positive for starch using Lugol. This corresponds to the initial phenotype for the mutation when seen in the *pls* mutant background, without the shortened root phenotype typical of the *pls* mutant (Casson and Lindsey, 2006). The use of 2,4 – D in

itself causes a shortened root phenotype by reducing cell production rate (Rahman et al., 2007) but is not as extreme as that seen in *pls.*

The change in cell identity has previously been documented (Casson and Lindsey, 2006), on rare occasions the phenotype is lethal, but mostly manifests as the swollen hypocotyl. Although abnormal morphology is most commonly found at the hypocotyl-root junction, it may be present anywhere along the length of the hypocotyl. The changes in cell identity were examined using SEM in the *pls* background where the epidermal cells were found to be much smaller and flatter than the wildtype (Casson and Lindsey, 2006).

Phytogel as a substitute for agarose was also shown to increase the penetrance of the *tnp* mutant. The phytogel caused smaller seedlings and a much more exaggerated 2,4-D phenotype, which may have been a result of mechanical impedance, due to a combined ethylene and auxin effect (Okamoto et al., 2008).

An interesting result of using 2, 4-D to increase the penetrance in the *tnp* mutant was the apparent induction of starch production in the wildtype hypocotyl (Figure 8). The starch accumulation appears to be to a lesser extent, but it is hard to quantify from image analysis alone. The phenotype did not exhibit such a swollen hypocotyl as seen in the *tnp* mutant, but starch granules were clearly present throughout the hypocotyl and in cells throughout the mature embryo. It has previously been shown that in *Arabidopsis,* storage molecules are mostly stored within the embryo (Nguyen et al., 2015) rather than between the embryo and endosperm. As a result, it is possible to observe starch granules throughout the wildtype embryo (Figure 7) before germination. It is, therefore, possible that starch observed in the 7 day old wildtype seedlings is just an embryonic carry-over. This could explain why the starch appears much less dense than that seen in *tnp,* but not why it is concentrated in the hypocotyl.

Another possibility is that the auxin is mediating the effect through the *LEC* pathway. Auxin plays an important role in plant development and is highly active through embryogenesis.

When the *tnp* mutant was first identified, the phenotype was correlated to increased auxin responsiveness by observation of the IAA2: GUS reporter (Casson and Lindsey, 2006). Despite this, when investigated, *LEC1* transcription levels appeared unaltered in response to auxin. When Geneinvestigator (Hruz et al., 2008) was used to determine if *LECs* showed positive regulation in direct response to auxin, there were also no significant results. It has previously been observed that *FUS3* is upregulated by auxin and is a positive regulator of ABA levels (Gazzarini et al., 2004). This is why it is important to study *FUS3* expression in the future. When the starch producing wildtype was examined for increased *LEC1* or *LEC2* expression there was no positive result. This is not particularly surprising as auxin has been shown to suppress *LEC2* expression (Casson and Lindsey, 2006). It is possible that this effect is being mediated by *FUS3* or an aspect of the *FUS3* pathway. It is also possible that we are simply seeing a remnant of starch accumulation in the embryo. ABA levels are relatively high during maturation when starch is accumulated and drop to low levels before germination (Baybrook & Harada, 2008). Another possibility is that auxin is suppressing the breakdown of the starch which is why granules are observed throughout the seedling. This could cause a reduced amount of biochemical energy available for growth and, therefore, a shortened phenotype such as the one seen in 2,4–D. This is, however, speculation and requires further analysis.

## 4.2 Sectional analysis

### 4.2.1 General trends

In each *Brassica* species tested, a unique time course was observed throughout its life cycle in respect to starch and lipid concentrations. Within this thesis *B. napus var. napobrassica* was used to illustrate these changes to prevent unnecessary replication of figures. This represents the general trends seen throughout the *Brassicas* where any major deviations are noted within the text. It is important to understand the changes taking place within the tissue in order to correlate with the *LEC* expression profiles. Within the *Brassicas* we hypothesised

that there would be a correlation between starch and lipid accumulation and expression levels of the *LEC* genes.

Starch was present in the 7 day section for each *Brassica* located around the central vascular tissue (Figure 9, Week1). This was unexpected, as morphologically the hypocotyl was not expressing any storage organ characteristics. Large amounts were not observed and the granules were confined to the exterior concentric ring of the vascular tissue. It was later confirmed that this characteristic is not unique to *Brassicas*, which exhibit swollen storage organs throughout their lifetime. A similar starch pattern was observed within *B.napus var. napus* (Figure 9, A) which suggests it is not an early starch accumulation mechanism for later formation of the swollen structure. The starch is also located externally to xylem where starch accumulation takes place in *B .rapa* (BiologyDiscussion, 2016). Between week 2 and week 4 little hypocotyl expansion is observed, but the area of vascular tissue is seen to grow to a proportionately large extent compared to stem area growth, replacing much of the ground tissue originally seen. Starch accumulation is first seen in week 4 externally to the xylem where the hypocotyl is still relatively small.

Week 5 appears to be the tipping point where hypocotyl diameter doubles and starch is accumulated uniformly across the xylem. After this point there is no real morphological change in the structure or starch distribution, the hypocotyl simply expands. Week 12 is the exception where a loss of starch is observed from the centre of the vascular tissue (Figure 9). This was thought to be due to structural modifications as the swollen structure appeared much denser and harder to section. As the tissue seemed structural the presence of lignin wastested for. Lignin is an important structural material in the support of tissues of vascular plants and fills the spaces in the cell wall especially in the xylem (Martone et al., 2009). It has several structural and hydrophobic properties that help in water absorption, but as a plant ages the amount of lignin in the cell walls increases, rendering them harder to digest (Russel,

1947). Phloroglucinol staining was not able to show a presence of lignin within the mature *B. napus* so it remains uncertain what this component is (Figure 7).

The fats and lipids show a much simpler profile to the starch, and do not seem to correlate as much with the hypocotyl expansion. After 14 days there is an immediate presence detected by the Fat Red (Figure 9, B) that seems to drop off before starch accumulation occurs. Spatially it is located in a very similar area to where the starch is found, but visually it is hard to quantify how much is present, and was quantified using gas chromatography (discussed below).

### 4.2.2 Variation between Brassicas

*Brassica rapa var. pt* was very similar to the profile seen within *B. napus*. Xylem expansion is observed over the same time period with striation of starch granules throughout the hypocotyl. After 5 weeks hypocotyl expansion and starch accumulation begin, but form a much less uniform staining pattern (Figure 11). Starch is not present between the cambium and the epidermis. This is consistent with previous experiments (BiologicalDiscussion, 2016). *B.rapa var. snowball* was much the same as *B. rapa var. pt,* except it was much smaller and was the only *Brassica* to exhibit lignin in the mature form. *B.oleracea* was the most unusual out of any of the varieties examined as it did not produce any significant quantities of starch. The swollen structure of *B. oleracea* did not appear to be derived from hypocotyl tissue, but instead the stem. Unlike *B. napus* or *B. rapa,* the swollen structure appeared to grow on the surface of the soil with the hypocotyl acting as an intermediary between this and the root.

*Raphanus sativus* was investigated due to its similar swollen structure in the hypocotyl, but belongs to a different genus. Initially development starts out very similarly to *B.napus*, but the mature form differs the most out of all of the examined varieties. *R.sativus* had the largest seed and embryo and as a result grew the fastest. The vascular expansion that was a precursor to hypocotyl expansion, in *B. napus,* happened much more rapidly in *R. sativus*

taking only two weeks (Figure 9, B). This developmental step is still a good indicator of when hypocotyl expansion occurs. Starch constitutes much less of the hypocotyl in *R. sativus* and is only present in small colonies in concentric rings in the outer xylem. Similarly to *B. napus* the mature form has a starch negative centre that did not stain positive for lignin. It is worth noting that by eight weeks *R. sativus* would no longer be edible and has effectively been left to seed. Much of its resources, therefore, go into stem and seed production (O'Hare, Wong and Force, 2009). Fats and lipids follow a very similar profile to *B.napus* and unlike starch, are present throughout the hypocotyl.

### 4.3 *LEC* expression in *Brassicas*

We have been able to demonstrate that *LEC* expression does take place outside of embryogenesis in *Brassicas.* Expression is not limited to the hypocotyl and was shown to be present throughout the organism. Levels were observed in the leaves and have previously been observed in the root and petiole (Tilley E., *Unpublished data, University of Durham*). The presence of *LEC* in non-hypocotyl tissue raises the question:"Why is there only a phenotype within the hypocotyl?" This once again goes beyond the scope of this thesis and is a question for further research.

The *LEC1* expression profiles were very similar across all of the examined varieties (Figure 16). There does seem to be a tight correlation between *LEC1* expression and the hypocotyl expansion observed in the sectional series (Figure 9). There is also a surprising amount of variation within the leaf, but we have no detectable morphological change associated with this. It would appear that there is a large expression peak one week before the hypocotyl development as a storage organ appears to begin. This then drops off and is superseded by *LEC2* expression after five weeks (Figure 18). It has previously been demonstrated in the literature that *LEC1* is able to regulate *LEC2* expression (To et al., 2006; Baybrook and Harada, 2008) and these profiles would appear to indicate that *LEC1* expression is followed by *LEC2*. *LEC2* has also been demonstrated to induce other *LEC* expression. Ectopic *LEC2* over expression in castor beans has been shown to induce accumulation of five major

transcription factor genes, *LEC1*, *FUS3*, *ABI3* and *LEAFY COTYLEDON1–LIKE (L1L)* which are involved in regulating fatty acid biosynthesis (Kim et al., 2013). This was shown to induce the accumulation of seed specific fatty acids such as eicosenoic acid (20:1) which was observed in the *tnp* mutant and *B. napus* leaf. We have observed wild type expression of *LEC* in vegetative tissue at the same time as we are observing accumulation of fatty acids and starch. Accumulation of seed fatty acids is a phenotype of ectopic expression in both *Arabidopsis* (Casson and Lindsey, 2006) and castor beans (*Kim et al., 2013*) so it would therefore seem possible that it is LEC proteins that are at least partly responsible for the storage organ development in *Brassicas*. Furthermore it would appear that in the young seedling, *LEC1* is the initial gene responsible for induction of hypocotyl expansion and starch accumulation. *LEC1* is then superseded by *LEC2* which is maintained at high levels until maturity. Due to the large amount of known crosstalk (To et al., 2006; Baybrook and Harada, 2008) it is not known whether *LEC1* is repressing *LEC2*, or causes its activation resulting in itself being regulated.

*B. oleracea* has relatively little *LEC2* expression in comparison to the other *Brassica*s, and unlike the other *Brassicas* shows an almost complete absence of starch in its swollen body (Figure 11, C). This morphology is shared by *R.sativus* which has comparatively low levels of *LEC2* throughout its life cycle, except for a single peak around five weeks. This would indicate that *LEC2* is intrinsically linked to starch accumulation in the hypocotyl as *LEC1* levels are very similar to the profiles observed in the other *Brassicas*. This suggests that *LEC2* is independently responsible for starch accumulation or has a paired effect with *LEC1*. It is also highly likely that *FUS3* plays some role due to its interactions with other *LECs* (Lotan et al., 1998; Santos Mendoza et al., 2005; Casson and Lindsey, 2006; Stone et al., 2008), but this could not be explored in the current study.

## 4.4 Fatty acid analysis

### 4.4.1 Seeds

It has previously been remarked that the *tnp* mutant showed no morphological differences with wildtype in the embryo (Casson and Lindsey, 2006). This suggests that any defect develops after germination. Although this observation of the embryo is true there is a *tnp* mutant seed phenotype where the volume and fatty acid content of the seed have increased (Figure 19). The *tnp* mutant seeds were 1.4x the volume of the wildtype counterpart and showed increased compositions of fatty acids 16:0, 18:2, 18:3 and 20:1 (Figure 21). Several of these compounds were identified in vegetative tissue when *LEC2* is ectopically expressed (Kim et al., 2013).

The original *tnp* mutant seeds were caused by an upstream deletion in the *LEC1* gene promoter region, whilst the seeds used in this thesis were complete *LEC1* genes driven by the strong CaMV35S promoter, without the *pls* background. It is not known if the original seeds exhibited this increase in size or fatty acid weight. *LEC1* is normally repressed in both vegetative and embryonic tissue by *PKL*. *PKL* is a gene that encodes a CHD3 chromatin remodelling factor, that forms part of a NuRD histone deacetylase complex (Ogas et al., 1999; Braybrook and Harada, 2008; Verdier and Thompson, 2008; Zhang and Ogas, 2009). It has been hypothesised that histones bound to the promotor region can be targeted for deacetylation by the NuRD complex or could contain binding regions for other transcriptional regulators (Casson and Lindsey, 2006). With complete loss of the promoter region it is not surprising that we see an increase in products known to be associated with the *LEC1* pathway.

When the *tnp* mutant seedling is analysed for fatty acids, we see the same fatty acid peaks that increased in the seed; 16:0, 18:2, 18:3 and 20:1 (18:2 and 18:3 are the fatty acids we see present in high concentrations in the *Brassica* hypocotyls).

The *Brassica* seeds were analysed to give a comparison to the adult tissue. Relatively similar profiles were seen across all varieties with large amounts of erucic acid (22:1) that was not present in such concentrations in *Arabidopsis.* The name 'erucic' pertains to *Eruca* which is a genus of flowering plant in the family *Brassicaceae,* of which *Brassica* is a genus. In previous studies it has been shown to constitute more than 50% of the seeds' fatty acid by weight in many *Brassicas* (Ahuja et al., 1987). Erucic acid has been bred out of industrial rapeseed (LEAR) due to studies that show a toxic effect on the heart in high enough doses, though this link has not been established in humans (Fsanz, 2003). It has been shown that mutations in *FATTY ACID ELONGATION1* (*FAE1*) lead to dramatic decreases of seed erucic acid content in *Arabidopsis* (Cao et al., 2010). This is particularly interesting as we did not observe large quantities of erucic acid in the *tnp* mutant, but did see large amounts in the other *Brassicas*. a significant increase in erucic acid in the *tnp* mutant was observed, but not of the same magnitude as the other FAs identified. In fact, ectopic expression of *LEC2* has been shown to cause increased expression of *FAE1* and induce the accumulation of triacylglycerol's (Kim et al., 2013).

It is feasible that the low amount of erucic acid in the *tnp* mutant seedling is due to a lack of *LEC1* repression. LEC1 has been demonstrated to mediate *LEC2* so it is possible we are seeing *FAE1* activation via this interaction. Also erucic acid was not observed in large quantities in the *Brassica* adult hypocotyl which is surprising as large amounts of *LEC2* expression were observed. There was a high amount of background variation in the gas chromatography results, so it is possible that any peak of erucic acid was lost. At the time it was not known it would be a peak of interest.

The fatty acid 16:0 (palmitic or hexadecanoic acid) is the most common saturated fatty acid found in animals, plants and microorganisms (Gunstone, Harwood and Dijkstra, 2007). It is, therefore, not surprising that we found this in all plant tissues studied.

When *Brassica* seeds are compared to *Arabidopsis*, approximately the same profile is observed with some variations. *Brassica*s were shown to contain a significantly higher proportion of the fatty acid 18:1 (oleic acid or elaidic acid). These are fatty acids in the omega-9 family which are common components of vegetable oil (Ahuja et al., 1987). Other members of this family include erucic acid, which was observed in high concentrations, and gondoic acid (20:1). Gondoic acid was one of the three fatty acids which were shown to increase in the *tnp* mutant seed.

The other two fatty acids observed to increase in the *tnp* mutant were 18:2 and 18:3. These are most likely linoleic acid and α-linolenic acid respectively. Linoleic acid (LA) is an essential fatty acid and its name is derived from the Greek 'linon' for flax. 'Oleic' meaning related to or derived from olive oil, or of relating to oleic acid. If the double bond is saturated in linolenic acid, oleic acid is formed (Burr and Burr, 1981). LA is a member of the omega-6 fatty acid family. LA has many industrial applications including uses in oil paints, varnishes and beauty products due to its beneficial effect on the skin (Darmstadt et al., 2007). It is a normal component of many seed, vegetable and nut oils (Bur and Bur, 1930).

α-Linolenic acid (ALA) is also an essential fatty acid. It is a member of the omega-3 fatty acid family predominantly found in seeds and vegetable oils (Beare-Rogers, Dieffenbacher and Holm, 2001). ALA has also been shown to be present in the thylakoid membranes in the leaves of *Pisum sativum* (Chapman, De-Felice and Barber, 1983). Regardless of this the best source of ALA remains from seeds, most seeds however are richer in LA with the exception of flax and chia seeds. ALA is relatively more susceptible to oxidisation which is why it is commonly hydrogenised in commercial use (Caswell, 2009). This, however, poses a potential health risk as homogenisation causes modification of an unsaturated aspect of ALA to become a trans-fat (Caswell, 2009).

### 4.4.2 Hypocotyl development

When the hypocotyl of *B. napus* was examined for fatty acids, large amounts of typically seed derived FAs were observed (Figure 25). These included ALA and LA, which were shown to dramatically increase in the *tnp* mutant, as well as oleic acid which can be derived from LA. Erucic acid was also present, which as previously discussed can be observed in vegetative tissue when *LEC2* is ectopically expressed (Kim et al., 2013). There were also large amounts of ALA, LA and erucic acid present within *B. napus* leaves, which is particularly interesting as these are considered seed storage products. This is consistent with the observed *LEC* expression within non hypocotyl tissue. Here we are proposing that there is an apparent effect caused by the ectopic *LEC* expression in a measurable form. Since it has previously been demonstrated that these two are linked, it is considered that there is a mechanistic link. Regardless of the expression in the leaves, the general fatty acid content of the hypocotyl was higher on all accounts with the exception of erucic acid. This further supports that *LECs* are being recruited for storage organ formation.

A similar effect was observed in *B .oleracea* where the same highlighted fatty acids were observed within the hypocotyl (Figure 26). When the percentage change from the seed is observed in both *B. napus* and *B. oleracea* there is a significant increase in the percentage composition of ALA (18:2). As this is a major constituent of all of the seeds observed (*Arabidopsis* and *Brassica*) it is particularly significant that we are seeing an increase from seed levels. This highlights the specific accumulation of what is known to be seed oil. Relatively little change in ALA is also observed, further highlighting the relative levels of these compounds present within the hypocotyl. Erucic acid is the only major highlighted fatty acid not shown to be present in high levels within the hypocotyl. Regardless, it is still present and is normally only observed within the seed.

Erucic acid has been demonstrated to be linked to *FAE1* transcript levels, and like *LECs* this is rarely expressed in vegetative tissue. In *Arabidopsis* it has been shown that transcript

levels are exclusively found within the embryo (James et al., 1995, Murata et al., 2003). *FAE1* is linked to the early torpedo stage embryo 4-5 days after flowering. The transcript levels reach their peak 9-11 days after flowering, the timing of which coincides with the period of major storage lipid accumulation. The promoter is known to being highly embryo specific (Mureta et al., 2003). The regulation of *FAE1* has been explored in embryonic and vascular tissues of *B.napus var. napus* which contains two homologous genes *Bn-FAE1.1* and *Bn-FAE1.2* (Chiron et al., 2015). The proximal regions of the two promotors exhibit strong sequence identity that suggests transcriptional control is mediated by this region. This contains putative cis-element characteristics of those found in the promoters of genes expressed in embryo and endosperm (Chiron et al., 2015). It is not currently known how *LEC* transcription is achieved in vegetative *Brassica* tissue as it is usually repressed by *PKL*. It is therefore possible that *FAE1* repression is still taking place in vegetative *Brassica* tissue regardless of *LEC* expression.

Higher levels of fatty acids were observed in the 7 day old *B.napus* seedling compared to the mature hypocotyl. This could be related to the much denser structure of the hypocotyl, relative levels of water and structural material and increased difficulty of extraction. Regardless of this, ectopic expression was observed in both vegetative tissues.

### 4.5 Future research

There are several possible avenues of research to follow on from this project. In particular, further analysis of *FUS3* should be carried out in both the *tnp* mutant and *Brassicas.* If transcription is present, this will support that the storage organ development is *LEC* derived.

Further quantitative analysis of lipids should be carried out throughout the *Brassica sp.* lifecycles in order to support the sectional series and provide further comparison to *LEC* transcription levels. Starch quantitative analysis should also be examined alongside lipid extraction in order to give numerical comparisons.

In order to determine whether the starch accumulation observed in *Col 0* is auxin dependent, RNA seek should be carried out in order to examine exactly why this effect is being observed.

Another interesting avenue of future research would be to analyse the spatial expression of embryonic genes in vegetative tissues of *Brassica*. This would involve cloning the homologous promotors for *LEC1*, *LEC2*, *FUS3 MDF* and other embryonic genes of interest, fuse them to the GUS reporter gene and produce transgenic plants. This will allow a visual representation of where the genes of interest are being expressed throughout the life cycle of the test species.

This should be paired with exploring why LEC expression is observed in vegetative *Brassica* tissue, whether it is related to a deletion in the promotor region such as seen in the *tnp* mutant, or caused by a further regulatory process.

### 4.6 Concluding remarks

We were able to demonstrate that LEC expression is observed in vegetative tissue which coincides with the accumulation of starch and lipids. Specifically lipids that are known to present in high levels in embryonic tissue and which are shown to be present when LECs are expressed ectopically in other plants. This strongly suggests that LECs have been recruited in crop evolution and selection to form the swollen storage organs observed in *Brassicas.* As these storage organs contain materials that are more readily used as bioethanol pre cursors, this could have potential value in the biofuel industry, allowing the design of novel crops that could sequestrate products that are much more easily/readily processed than current second generation biofuels such as cellulose. This could increase the bioethanol yield per hectare of crops whilst reducing the consumption of food crops for non-consumable uses.

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# 6 Appendices

## Appendix 1 – Plant Materials

| Plant Materials Used | |
|---|---|
| Wild-type *Arabidopsis (Columbia 24)* | |
| *Arabidopsis tnp* line 24 | |
| Johnsons turnip seeds: | *Brassica rapa* – Varieties ‘Purple top’ and ‘Snowball’ |
| Fothergill turnip seeds: | *Brassica napus* – Variety ‘Helenor’ |
| | *Brassica oleracea* – Variety ‘Olivia’ |
| | *Raphanus sativus* – Variety ‘Globe’ |
| Oil seed rape : | *Brassica napus* – Variety ‘Belinda’ |
| J. Arthur Bower’s John Innes no.3 Soul based Compost | |

**Appendix 2 – Chemical Materials**

| Chemical Materials Used | |
|---|---|
| **Company** | **Chemical(s)** |
| Invitrogen™ | SuperScript™ III First-Strand Synthesis System for RT-PCR - OligodT, 10X RT Buffer, 25mM $MgCl_2$, 0.1 mM DTT, 10mM dNTP RNase OUT™, SuperScript™III RT.<br>Select-Agar. |
| Qiagen | RNeasy Plant RNA Extraction Mini Kit. |
| Bioline | PCR Mastermix Components – 10X Reaction Buffer, 25mM $MgCl_2$, Taq Polymerase, Hyperladder I and IV, dNTPs.<br>Agarose Multi-Purpose, 6X loading dye. |
| Sigma Aldrich | Murashige and skoog salts.<br>Sucrose.<br>Lugol solution.<br>Fat Red stain<br>Spectrum™ Plant Total RNA Kit<br>On-Column DNase I Digestion Set<br>Tween – 20<br>2,4-Dichlorophenoxyacetic acid<br>Pholoroglucinol |

| | |
|---|---|
| ICN Biomedicals Ltd | Ethidium Bromide. |
| Mexcel Dispoco Ltd | Sodium hypochlorite solution – bleach. |
| VWR international Ltd | Ethanol. |
| Lab 1004 Stock | Liquid Nitrogen.<br>Hoyer's solution.<br>Histoclear.DNA extraction buffer. |

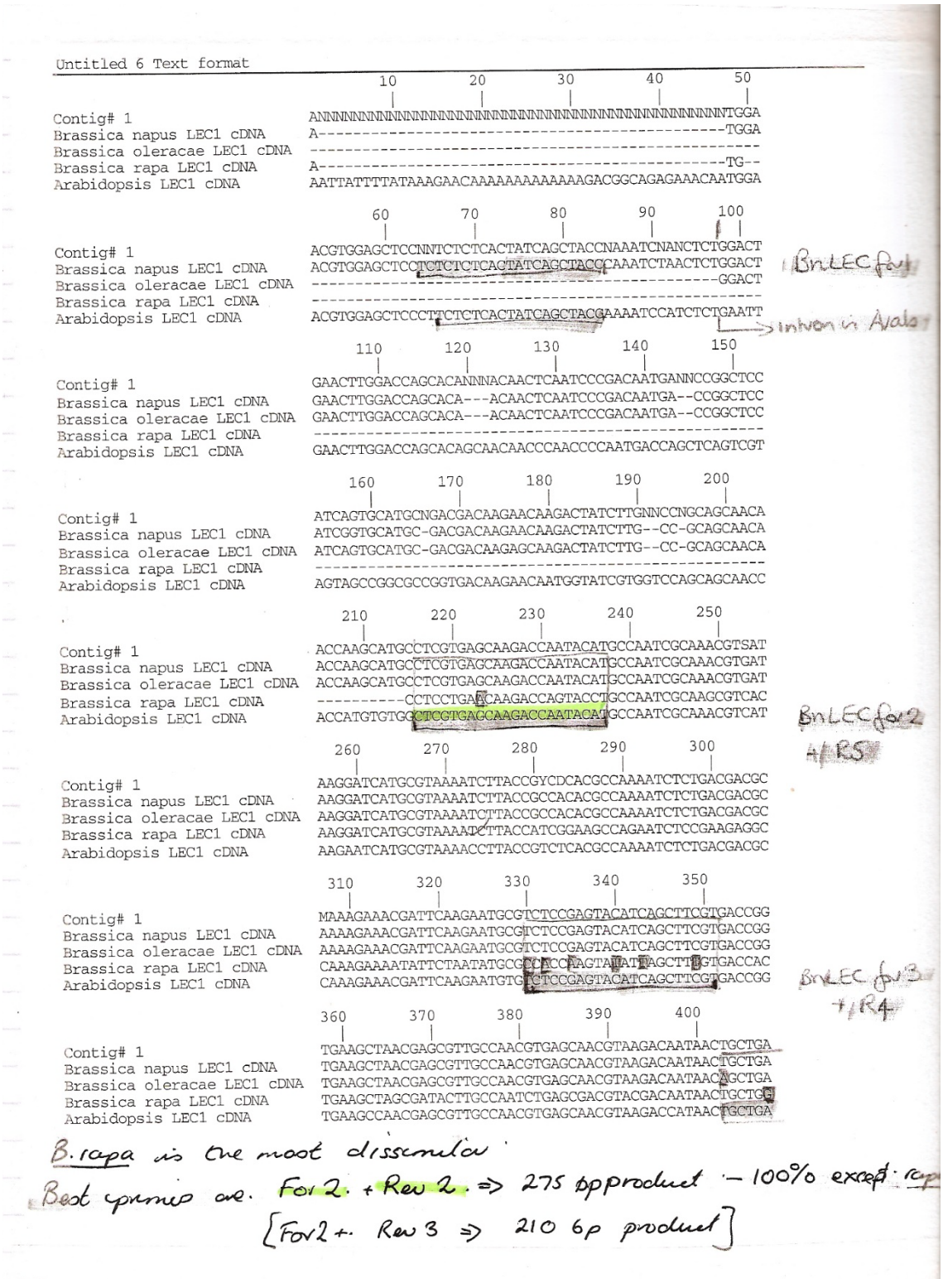
**Appendix 3 – Experimental Materials used**

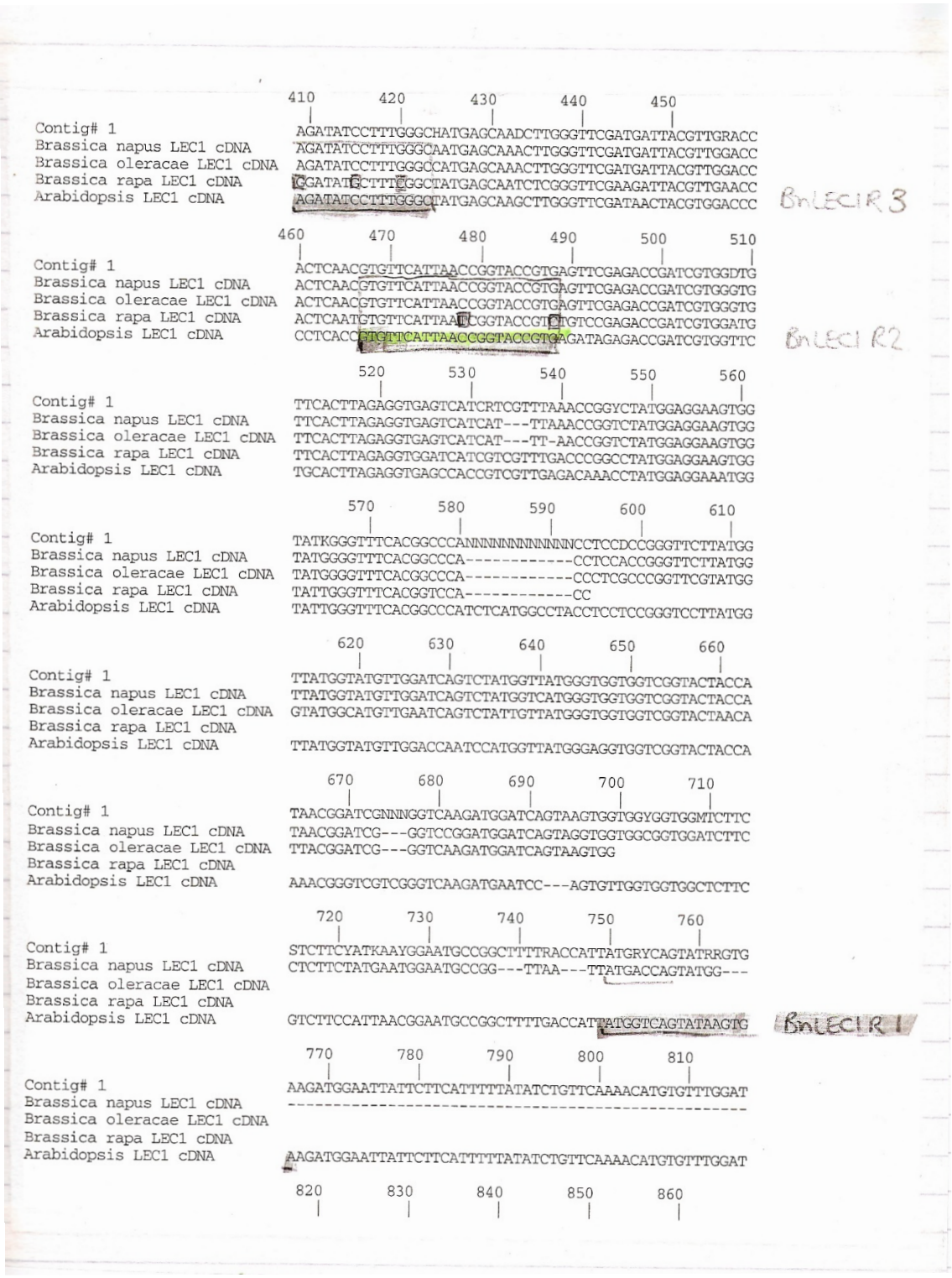
| Experimental Materials Used | |
|---|---|
| **Manufacturer/Model** | **Equipment/Use** |
| Gilson | Pipette |
| BioRad | Electrophoresis trays and power packs |
| LabNet Spectrafuge 16M | Centrifuge |
| Eppendorf Concentrator 5301 | Centrifuge |
| Epson 1680 pro flathead scanner | Scanner |
| Syngene 'InGenius L' | UV Illuminator |
| Olympus SZH10 | Light Microscope |
| QICAM QIMAGING | Camera used in conjunction with 'OpenLab' 3.1.1 software (Improvision) for specimen photographs |
| Spectrophotometer - Nanodrop | RNA purity measure using Nucleic Acid – ND-1000v35.2 |

| | |
|---|---|
| Oligo V4.02 and Amplify V1.2 | Primer design programmes |
| Sigma Aldrich | Tip one, filter pipette tips<br><br>Petri Dish square, L × W 120 mm × 120 mm |

## Appendix 4 Brassica Orthologue alignments

*LEC1 cDNA Bioinformatic Sequences for Arabidopsis, Brassica napus, B. rapa and B. oleracea*




410 420 430 440 450
Contig# 1
Brassica napus LEC1 cDNA
Brassica oleracae LEC1 cDNA
Brassica rapa LEC1 cDNA
Arabidopsis LEC1 cDNA
AGATATCCTTTGGGCHATGAGCAADCTTGGGTTCGATGATTACGTTGRACC
AGATATCCTTTGGGCAATGAGCAAACTTGGGTTCGATGATTACGTTGGACC
AGATATCCTTTGGGCCATGAGCAAACTTGGGTTCGATGATTACGTTGGACC
CGATATGCTTTCGGCTATGAGCAATCTCGGGTTCGAAGATTACGTTGAACC
AGATATCCTTTGGGCTATGAGCAAGCTTGGGTTCGATAACTACGTGGACCC
BnLECIR3
460 470 480 490 500 510
Contig# 1
Brassica napus LEC1 cDNA
Brassica oleracae LEC1 cDNA
Brassica rapa LEC1 cDNA
Arabidopsis LEC1 cDNA
ACTCAACGTGTTCATTAACCGGTACCGTGAGTTCGAGACCGATCGTGGDTG
ACTCAACGTGTTCATTAACCGGTACCGTGAGTTCGAGACCGATCGTGGGTG
ACTCAACGTGTTCATTAACCGGTACCGTGAGTTCGAGACCGATCGTGGGTG
ACTCAATGTGTTCATTAACCGGTACCGTGTGTCCGAGACCGATCGTGGATG
CCTCACCGTGTTCATTAACCGGTACCGTGAGATAGAGACCGATCGTGGTTC
BnLECIR2
520 530 540 550 560
Contig# 1
Brassica napus LEC1 cDNA
Brassica oleracae LEC1 cDNA
Brassica rapa LEC1 cDNA
Arabidopsis LEC1 cDNA
TTCACTTAGAGGTGAGTCATCRTCGTTTAAACCGGYCTATGGAGGAAGTGG
TTCACTTAGAGGTGAGTCATCAT---TTAAACCGGTCTATGGAGGAAGTGG
TTCACTTAGAGGTGAGTCATCAT---TT-AACCGGTCTATGGAGGAAGTGG
TTCACTTAGAGGTGGATCATCGTCGTTTGACCCGGCCTATGGAGGAAGTGG
TGCACTTAGAGGTGAGCCACCGTCGTTGAGACAAACCTATGGAGGAAATGG
570 580 590 600 610
Contig# 1
Brassica napus LEC1 cDNA
Brassica oleracae LEC1 cDNA
Brassica rapa LEC1 cDNA
Arabidopsis LEC1 cDNA
TATKGGGTTTCACGGCCCANNNNNNNNNNNNCCTCCDCCGGGTTCTTATGG
TATGGGGTTTCACGGCCCA----------CCTCCACCGGGTTCTTATGG
TATGGGGTTTCACGGCCCA----------CCCTCGCCCGGTTCGTATGG
TATTGGGTTTCACGGTCCA----------CC
TATTGGGTTTCACGGCCCATCTCATGGCCTACCTCCTCCGGGTCCTTATGG
620 630 640 650 660
Contig# 1
Brassica napus LEC1 cDNA
Brassica oleracae LEC1 cDNA
Brassica rapa LEC1 cDNA
Arabidopsis LEC1 cDNA
TTATGGTATGTTGGATCAGTCTATGGTTATGGGTGGTGGTCGGTACTACCA
TTATGGTATGTTGGATCAGTCTATGGTCATGGGTGGTGGTCGGTACTACCA
GTATGGCATGTTGAATCAGTCTATTGTTATGGGTGGTGGTCGGTACTAACA
TTATGGTATGTTGGACCAATCCATGGTTATGGGAGGTGGTCGGTACTACCA
670 680 690 700 710
Contig# 1
Brassica napus LEC1 cDNA
Brassica oleracae LEC1 cDNA
Brassica rapa LEC1 cDNA
Arabidopsis LEC1 cDNA
TAACGGATCGNNNGGTCAAGATGGATCAGTAAGTGGTGGYGGTGGMTCTTC
TAACGGATCG---GGTCCGGATGGATCAGTAGGTGGTGGCGGTGGATCTTC
TTACGGATCG---GGTCAAGATGGATCAGTAAGTGG
AAACGGGTCGTCGGGTCAAGATGAATCC---AGTGTTGGTGGTGGCTCTTC
720 730 740 750 760
Contig# 1
Brassica napus LEC1 cDNA
Brassica oleracae LEC1 cDNA
Brassica rapa LEC1 cDNA
Arabidopsis LEC1 cDNA
STCTTCYATKAAYGGAATGCCGGCTTTTRACCATTATGRYCAGTATRRGTG
CTCTTCTATGAATGGAATGCCGG---TTAA---TTATGACCAGTATGG---
GTCTTCCATTAACGGAATGCCGGCTTTTGACCATTATGGTCAGTATAAGTG
BnLECIR1
770 780 790 800 810
Contig# 1
Brassica napus LEC1 cDNA
Brassica oleracae LEC1 cDNA
Brassica rapa LEC1 cDNA
Arabidopsis LEC1 cDNA
AAGATGGAATTATTCTTCATTTTTATATCTGTTCAAAACATGTGTTTGGAT
----------
AAGATGGAATTATTCTTCATTTTTATATCTGTTCAAAACATGTGTTTGGAT
820 830 840 850 860

LEC2 Brassica alignment

```
Bra032890               ATGGATAACTTCTTGCCCTTTTCCTCTTCTAACGCAAACTTTGTCCAAGAACTCTCAATG
Bra030087               ATGGATAACTTCTTGCCCTTTTCCTCTTCTAACGCAAACTCTGTCCAAGAACTCTCCATG
Bol041825               ATGGATAACTTCTTGCCCTTTTCCTCTTCTAACGCAAACTCTGTCCAAGAACTCTCCATG
GSBRNA2T00056765001     ATGGATAACTTCTTGCCCTTTTCCTCTTCTAACGCAAACTCTGTCGAAGAACTCTCAATG
GSBRNA2T00157904001     ATGGATAACTTCTTGCCCTTTTCCTCTTCTAACGCAAACTCTGTCCAAGAACTCTCCATG
GSBRNA2T00089892001     ATGGATAACTTCTTGCCCTTTTCCTCTTCTAACGCAAACTCTGTCCAAGAACTCTCCATG
                        **************************************** **** **********.***

Bra032890               GATCTTAACAACAATCGCTCACGCTTATCAACGTTCCCTACTTATGATCATCATCATCAG
Bra030087               GATCTTAACAAGAATCGCTCGCACTTCTCCA--------------------------TG
Bol041825               GATCTTAACAAGAATCGCTCGCACTTCTCCA--------------------------TG
GSBRNA2T00056765001     GATCTTAACAACAATCGCTCACGCTTATCAACGTTCCCTACTTATGATCATCATCATCAG
GSBRNA2T00157904001     GATCTTAACAAGAATCGCTCGCACTTCTCCAT-------------------------G
GSBRNA2T00089892001     GATCTTAACAAGAATCGCTCGCACTTCTCCAT-------------------------G
                        *********** ********.*.***.**.*                          *

Bra032890               GCGCAGCCT---CGCTCGTTGCAACCCTACTCATATGTTGCATGTCCTGT---GGATCAG
Bra030087               GCGCAGCCTCAGCACTTGTTGCCGCCTTACTCGTACGTTGCATGTCCGGCACTTGATCAG
Bol041825               GCGCAGCCTCAGCACTTGTTGCCGCCTTACTCGTACGTTGCATGTCCGGTACTTGATCAG
GSBRNA2T00056765001     GCGCA---GCATCACTCGTTGCAACCCTACTCATACGTTGCATGTCCTGTG---GATCAG
GSBRNA2T00157904001     GCGCAGCCTCAGCACTTGTTGCCGCCTTACTCGTACGTTGCATGTCCGGTACTTGATCAG
GSBRNA2T00089892001     GCGCAGCCTCAGCACTTGTTGCCGCCTTACTCGTACGTTGCATGTCCGGCACTTGATCAG
                        *****       *.** *****..** *****.** *********** *     ******

Bra032890               ACGGCGGC-----------------------------TATGAATCCTCAGATCCCGGTT
Bra030087               ACGGGGACCATGAATCATCAGCCTCTTCACTCATCGGATGCTTTTCCTCAGATCCCGGTT
Bol041825               ACGGGGGCCATGAATCATCAGCCTCTTCACTCATCGGATGCTTTTCCTCAGATCCCGGTT
GSBRNA2T00056765001     ACGGCGGCTATGA---------------------------ATCCTCAGATCTCGGTT
GSBRNA2T00157904001     ACGGGGGCCATGAATCATCAGCCTCTTCACTCATCGGATGCTTTTCCTCAGATCCCGGTT
GSBRNA2T00089892001     ACGGGGACCATGAATCATCAGCCTCTTCACTCATCGGATGCTTTTCCTCAGATCCCGGTT
                        **** *.*                                  :********** *****

Bra032890               ACACAAACCGGAAGTGAGTTCGGTTCTCTGGTTTGTAATCCCGGTTTCGGACAAGCAAGA
Bra030087               GTACAAACCGGAGGTGAATTCGGCTATTTGGTTTGTAAGCCCGGTGTGAGGCAGGAACGA
Bol041825               GTGCAAACCGGAGGTGAATTCGGCTATTTGGTTTGTAAGCCCGGTGTGAGGCAGGAAAGA
GSBRNA2T00056765001     ATACAAACCGGAAGTGAGTTCGGTTCTCTGGTTTGTAATCCCGGTTTCAGACAAGCAAGA
GSBRNA2T00157904001     GTGCAAACCGGAGGTGAATTCGGCTATTTGGTTTGTAAGCCCGGTGTGAGGCAGGAAAGA
GSBRNA2T00089892001     GTACAAACCGGAGGTGAATTCGGCTATTTGGTTTGTAAGCCCGGTGTGAGGCAGGAACGA
                        . .*********.****.***** *.* ********** ****** * .*.**.*.*.**

Bra032890               GGTGGATTTCTTGATCCACACACGGCTAAGATGGCCAGGATCAACAGAAAGAAAGCGATG
Bra030087               GGTGGATTTCTTGATCCACACTCCACTAAGATGGCTAGGATCAACAGGAAGAAGGCGATG
Bol041825               GGTGGATTTCTTGATCCACACTCGACTAAGATGGCTAGGATCAACAGGAAGAAGGCGATG
GSBRNA2T00056765001     GGTGGATTCCTTGATCCACACACGGCTAAGATGGCCAGGATCAACAGAAAGAAAGCGATG
GSBRNA2T00157904001     GGTGGATTTCTTGATCCACACTCGACTAAGATGGCTAGGATCAACAGGAAGAAGGCGATG
GSBRNA2T00089892001     GGTGGATTTCTTGATCCACACTCCACTAAGATGGCTAGGATCAACAGGAAGAAGGCGATG
                        ******** ************:* .********** ***********.******.******

Bra032890               ATAAGATCAAGAAACAATTCTAGCCCTAACTCTAGTTCGAATGAGCTGGTTGGTTCAAGG
Bra030087               CTAAGATCAAGAAACAACTCTAACCCTAATTCTAGTTCGAATGAGTTGGTTGATTCAAGG
Bol041825               ATAAGATCAAGAAACAACTCTAACCTTAATTCTAGTTCGAATGAGTTGGTTGATTCAAGG
GSBRNA2T00056765001     ATAAGATCAAGAAACAATTCTAGCCCTAACTCTAGTTCGAATGAGCTGGTTGGTTCAAGG
GSBRNA2T00157904001     ATAAGATCAAGAAACAACTCTAACCTTAATTCTAGTTCGAATGAGTTGGTTGATTCAAGG
GSBRNA2T00089892001     CTAAGATCAAGAAACAACTCTAACCCTAATTCTAGTTCGAATGAGTTGGTTGATTCAAGG
                        .**************** ****.** *** *************** ******.*******

Bra032890               AGACAAGTGGTTCTTACCATGAAAAATAATGCCGAGATAGCAGCAAGGAAAGATCTCTAT
Bra030087               AGACAAGTGGCTCTTACCATGAAAAATAATGCCGAGATTGCTGCTAGAAAAGATTTTTAT
Bol041825               AGACAAGTGGCTCTTACCATGAAAAATAATGCCGAGATTGCTGCTAGAAAAGATTTTTAT
GSBRNA2T00056765001     AGACAAGTGGTTCTTACCATGAAAAATAATGCCGAGATTGCAGCAAGGAAAGATCTCTAT
GSBRNA2T00157904001     AGACAAGTGGCTCTTACCATGAAAAATAATGCCGAGATTGCTGCTAGAAAAGATTTTTAT
GSBRNA2T00089892001     AGACAAGTGGCTCTTACCATGAAAAATAATGCCGAGATTGCTGCTAGAAAAGATTTTTAT
                        ********** ****************************:**:**:**.****** * ***
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Bra032890               CGATATCCCTCATTCGATAACAAGAAGCTTAGGGTTTTGCTGGTGAAACACTTGAAGAAC
Bra030087               CGATTCTCCTCATTCGATAACAAGAAACTTAGGGTTTTGTTGGTGAAGCACTTGAAGAAC
Bol041825               CGATTCTCCTCATTCGATAACAAGAAACTTAGGGTTTTGTTGGTGAAGCACTTGAAGAAC
GSBRNA2T00056765001     CGATATTCCTCATTCGATAACAAGAAGCTTAGGGTTTTGCTGGTGAAACACTTGAAGAAC
GSBRNA2T00157904001     CGATTCTCCTCATTCGATAACAAGAAACTTAGGGTTTTGTTGGTGAAGCACTTGAAGAAC
GSBRNA2T00089892001     CGATTCTCCTCATTCGATAACAAGAAACTTAGGGTTTTGTTGGTGAAGCACTTGAAGAAC
                        ****:  ******************.************ *******.************

Bra032890               AGCGATGTTGGATCACTTGGGAGGATCGTTCTACCAAAGAGAGAAGCTGAAGGGAATCTT
Bra030087               AGCGATGTTGGGTCACTTGGGAGGATTGTTCTACCAAAGAGAGAAGCAGAAGGAAATCTT
Bol041825               AGCGATGTTGGGTCACTTGGGAGGATTGTTCTACCAAAGAGAGAAGCAGAAGGAAATCTT
GSBRNA2T00056765001     AGCGATGTTGGATCACTTGGGAGGATCGTTCTACCAAAGAGAGAAGCTGAAGGGAATCTT
GSBRNA2T00157904001     AGCGATGTTGGGTCACTTGGGAGGATTGTTCTACCAAAGAGAGAAGCAGAAGGAAATCTT
GSBRNA2T00089892001     AGCGATGTTGGGTCACTTGGAAGGATTGTTCTACCAAAGAGAGAAGCAGAAGGAAATCTT
                        ***********.********.***** ********************:*****.******

Bra032890               CCGGAGCTATCTACTAAAGAAGGAATGATAGTAGACATGAGAGATGCGGACTCTATGCAG
Bra030087               CCGGAGCTATCTGAGAAAGAAGGAATGGTATTAGAGATGAGAGATGTTGACTCTGTGCAG
Bol041825               CCGGAGCTATCTGATAAAGAAGGAATGGTATTACAGATGAGAGATGTTGACTCTGTGCAG
GSBRNA2T00056765001     CCGGAGCTATCTACTAAAGAAGGAATGATAGTAGAGATGAGAGATGCGGACTCTATGCAG
GSBRNA2T00157904001     CCGGAGCTATCTGATAAAGAAGGAATGGTATTACAGATGAGAGATGTTGACTCTGTGCAG
GSBRNA2T00089892001     CCGGAGCTATCTGATAAAGAAGGAATGGTATTAGAGATGAGAGATGTTGACTCTGTGCAG
                        ************.. ************.** ** * **********  ******.*****

Bra032890               AATTGG---------------TTCTGGTCCAATAACAAGAGCAGAATGTATGTCCTTGAA
Bra030087               TCTTGG---------------TACTGGTCCAATAACAAGAGCAGAATGTATGTCCTCGAA
Bol041825               TCTTGG---------------TACTGGTCCAATAACAAGAGCAGAATGTATGTCCTCGAA
GSBRNA2T00056765001     AATTGGTCTTTCAAATACAAGTTCTGGTCCAATAACAAGAGCAGAATGTATGTCCTTGAA
GSBRNA2T00157904001     TCTTGGTCTTTCAAATACAAGTACTGGTCCAATAACAAGAGCAGAATGTATGTCCTCGAA
GSBRNA2T00089892001     TCTTGGTCTTTCAAATACAAGTACTGGTCCAATAACAAGAGCAGAATGTATGTCCTCGAA
                        :.****              *:********************************** ***

Bra032890               AACACAGGACAATTTGTGACTGAAAAAAGAGTTGAGATTGGAGATTTTTTAACAATCTAC
Bra030087               AACACAGGAGAATTTGTGAAGAAAAATGGAGTATTGATGGGAGACTATCTAACAATCTAC
Bol041825               AACACAGGAGAATTTGTGAAGAAAAATGGAGTATTGATGGGAGACTATCTAACAATCTAC
GSBRNA2T00056765001     AACACAGGAGAATTTGTGGCTGAAAAAAGAGTTGAGATTGGAGATTTTTTAACAATCTAC
GSBRNA2T00157904001     AACACAGGAGAATTTGTGAAGAAAAATGGAGTATTGATGGGAGACTATCTAACAATCTAC

Bra030087               AACACAGGAGAATTTGTGAAGAAAAATGGAGTATTGATGGGAGACTATCTAACAATCTAC
Bol041825               AACACAGGAGAATTTGTGAAGAAAAATGGAGTATTGATGGGAGACTATCTAACAATCTAC
GSBRNA2T00056765001     AACACAGGAGAATTTGTGGCTGAAAAAAGAGTTGAGATTGGAGATTTTTTAACAATCTAC
GSBRNA2T00157904001     AACACAGGAGAATTTGTGAAGAAAAATGGAGTATTGATGGGAGACTATCTAACAATCTAC
GSBRNA2T00089892001     AACACAGGAGAATTTGTGAAGAAAAATGGAGTATTGATGGGAGACTATCTAACAATCTAC
                        ********* ********.. .****:.****: :*** ***** *:* ***********

Bra032890               GAGGACGAAAGCAAGAATCTCTACTTCTCCATAAGAAAGCACGCAGACAAACCAAATGAA
Bra030087               GAGGACGAAAGCAAGAATCTCTACTTCTCCATCAGAAAGCACCCACACAAACAAAATGAT
Bol041825               GAGGACGAAAGCAAGAATCTCTACTTCTCCATCAGAAAGCACCCACACAAACAAAATGAT
GSBRNA2T00056765001     GAGGACGAAAGCAAGAATCTCTACTTCTCCATAAGAAAGCACGCAGACAAACCAAATGAA
GSBRNA2T00157904001     GAGGACGAAAGCAAGAATCTCTACTTCTCCATCAGAAAGCACCCACACAAACAAAATGAT
GSBRNA2T00089892001     GAGGACGAAAGCAAGAATCTCTACTTCTCCATCAGAAAGCACCCACACAAACAAAATGAT
                        **********************************.********* ** ******.******:

Bra032890               GGAAGAGAAGATGAGTCGATGGAAGCCAACGACATGAACTTCTACGAAGATATTGCGTTT
Bra030087               GGAAGAGAGGATGAGTCGATGGAAGTTATCGAGATGAACTTCTATGAAGATATAATGTTT
Bol041825               GGAAGAGAGGATGAGTCAATGGAAGTCATCGAGATGAACTTCTATGAAGATATAATGTTT
GSBRNA2T00056765001     GGAAGAGAAGATGAGTCGATGGAAGCCAACGACATGAACTTCTACGAAGATATTGCGTTT
GSBRNA2T00157904001     GGAAGAGAGGATGAGTCAATGGAAGTCATCGAGATGAACTTCTATGAAGATATAATGTTT
GSBRNA2T00089892001     GGAAGAGAGGATGAGTCGATGGAAGTTATCGAGATGAACTTCTATGAAGATATAATGTTT
                        ********.********.*******  *:*** *********** ********:. ****

Bra032890               GATTTCATACCAAAAGATGAAGACGAAGATTCTATTGCAATGCTCATCGGAAATCTAAAT
Bra030087               GATTACATACCAAATGATGAAGAC---GATTCCATTGCAATGCTCCTCGGAAATCTAAAC
Bol041825               GATTACATACCAAATGGTGAAGAC---GATTCCATTGCAATGCTCCTCGGAAATCTAAAC
GSBRNA2T00056765001     GATTTCATACCAAAAGATGAAGACGAAGATTCTATTGCAATGCTCATCGGAAATCTAAAT
GSBRNA2T00157904001     GATTACATACCAAATGGTGAAGAC---GATTCCATTGCAATGCTCCTCGGAAATCTAAAC
GSBRNA2T00089892001     GATTACATACCAAATGATGAAGAC---GATTCCATTGCAATGCTCCTCGGAAATCTAAAC
                        ****:**********:*.*******   ***** ************.**************

Bra032890               GATCACTATCCCAACCCAAACAATCGTATGGACCTCCCAATCGATCTTCATCAGCATCAT
Bra030087               GAGCACTATCCCTACCCAAATGATCTTATGGATCTCACTGTCAATCTTGATCAGCATCAG
Bol041825               GAGCACTATCCCTACCCAAATGATATTATGGATCTCACTGTCGATCTTGATCAGCATCAG
GSBRNA2T00056765001     GATCACTATCCCAACCCAAACAATCTTATGGACCTCCCAATCGATCTTCATCAGCATCAT
GSBRNA2T00157904001     GAGCACTATCCCTACCCAAATGATATTATGGATCTCACTGTCGATCTTGATCAGCATCAG
GSBRNA2T00089892001     GAGCACTATCCCTACCCAAATGATCTTATGGATCTCACTGTCAATCTTGATCAGCATCAG
                        ** *********:******* .**. ****** ***.*:.**.***** **********
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Bra032890               CAAGCCACCTCA---TTGCCACCTGCGGATTACATGACCAATCCTCAGTATGGTGGTTCC
Bra030087               CAAGCCACCTCCTCGTCGCCACCTGCTGATCACATG-----------------------
Bol041825               CAAGCCACCTCCTCGTCGCCACCTGCTGATCACATG-----------------------
GSBRNA2T00056765001     CAAGCCACCTCCTCGTTGCCACCTGTGGATTACATGACCAATCCTCAGTATAGTGGTTCC
GSBRNA2T00157904001     CAAGCCACCTCCTCGTCGCCACCTGCTGATCACATGA----------------------
GSBRNA2T00089892001     CAAGCCACCTCCTCGTCGCCACCTGCTGATCACATGA----------------------
                        ***********.   * ********  *** *****

Bra032890               TCCAATGATCTCATGAGCTTTAACGACTTCGTATGGTGA
Bra030087               ---------------AGCTCGAACGATTTCTTATGGTGA
Bol041825               ---------------AGCTCGAACGATTTCTTATGGTGA
GSBRNA2T00056765001     TCCAATGATCACATGAGCTTTAACGACTTCGTATGGTGA
GSBRNA2T00157904001     ----------------GCTCGAACGATTTCTTATGGTGA
GSBRNA2T00089892001     ----------------GCTCGAACGATTTCTTATGGTGA
                                     ***  ***** *** ********
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Raphanus LEC1

```
RSG08196        ATGGACCAGCATAATAACTCAATCCCGGAAGCGACCGGCAACATCATTGCCTGCGACGAC
RSG45434        ------------------------------ATGACCGGCTCC---ATCAGTGCATGCGAC
                                              *******  *   **         ****

RSG08196        GACAAGAACAAGGTTATCGTTCAGCAGCAACAACCATGCATGGCTCGTGAACAAGACCAA
RSG45434        GATAAGAACAAGACTATCTTGCCGCAGCAACAACCAAGCATGCCTCGTGAGCAAGACCAA
                ** *********  **** * * ************** ***** ******* *********

RSG08196        TACATGCCAATCGCTAACGTCATAAGGATAATGCGTAGGACCTTACCGCCGCACGCCAAA
RSG45434        TACATGCCAATCGCAAACGTGATAAGGATCATGCGTAAAATCTTACCGCCTCACGCCAAA
                *************** ***** ******** *******  * ********* *********

RSG08196        ATCTCTGATGACGCCAAAGAAACGATTCAAGAATGCGTCTCCGAGTACATCAGCTTCGTT
RSG45434        ATCTCTGACGACGCCAAAGAAACGATTCAAGAATGCGTCTCCGAGTACATCAGCTTCGTG
                ******** ************************************************

RSG08196        ACCGGTGAAGCCAACGAGCGTTGCCAACGTGAGCAACGAAAGACCATAACTACTGAAGAC
RSG45434        ACCGGTGAAGCTAACGAGCGTTGCCAACGGGAGCAACGCAAGACAATAACTGCTGAAGAT
                *********** ***************** ******** ***** ****** *******

RSG08196        ATCCTTTGGGCTATGAGCAAGCTTGGTTTCGATGACTACGTTGGACCACTAAACGTGTTC
RSG45434        ATCCTTTGGGCGATGAGCAAGCTTGGGTTCGATGATTACGTTGGACCACTCAACTTGTTC
                *********** ************** ******** ************** *** *****

RSG08196        ATTAACCGGTACCGTGAGTTCGAGACTGATCGTGGGTGTTCACTTAGAGGTGAGTCATCG
RSG45434        ATTAACCGGTACCGTGAGTTCGAGACCGATCGTGGGTGTTCACTTAGAGGTGAATCTTCT
                ************************** ************************** ** **

RSG08196        TCGTTTAAACCGGCCTTTGGAGGAAGTGGTTTCGGTTTTCACCCCCCACCTCAAGGCCCA
RSG45434        TCGTTTAAACCTGTATATGGAGGAAGTGGTATAGGGTTTCATGGC------------CCA
                *********** *  * ************* * ** *****   *           ***

RSG08196        CATCCTCTTGGTCCTTATGGTTACGGTATGTTGGATCAGTCTATGGTTATGGGTGGTGGT
RSG45434        CCTCCCCCGGGTCCTTATGGTTACGGTATGTTGGATCAATCTATGGTTATGGGTGGTGGT
                * *** *  ****************************** **********************

RSG08196        CGGTACTACCAAAACGGATCGGGTCAGGATGGATCCGGTGGTGGTGGTGGTGGATCTTCG
RSG45434        CGGTACTACAATAACGGATCGGGTCAGGATGGATCCGTAGGTGGTGGT---GGATCTTCC
                ********* * *************************  *********   ********

RSG08196        TCTTCTGTGAATGGAATGCCGGTTTTTGACCAGTATGGTCAGTATAAGTGA---------
RSG45434        TCTTCTATTAATGGAATGCCGGTTTATGACCAGTATGTTGGTACTGTTGTCTCCGGCATC
                ****** * ***************** *********** *     *
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CLUSTAL O(1.2.1) multiple sequence alignment

Raphanus LEC2

RSG40475        ATGGATAACCTCTTGCCCTTTTCCTCTTCTAACGCAAACTCTGTCCAAGAACTCTCCATG
RSG50932        ATGGATAACTTCTTGCCCTTTTCCTCTTCTAACGCAAACTCTGTCGAAGAACTCTCAATG
                ********* ************************************ ********** ***

RSG40475        GATCTTAACAACAATCGCTCGCACTTGTCAACAATGGCGCAGCCTCACGACTTGTTGCCG
RSG50932        GATCTTAACAACAATCCCTCACGCTTCTCAACGCTCCCTACTTCTGATCATCATCATCAG
                ***************** *** * *** *****  *  *     ** *  *       * *

RSG40475        CCTTACTCGTACGTTGCATGTCCGGTACTTGATCAGACGGGGGCCATGAATCATCAGACT
RSG50932        GCGCAGCCTCACCC--CTTGTTGCCACCTTACTCAT-------ACGTTGCATCTCCTGTG
                 *  *  *  **    * ***      ***  ***         * *      **

RSG40475        TTTCACTTATCGGATGCTTTTCCTCACATATCGGTTGTGCAAACCGGAAGTGAATTCGGC
RSG50932        GATCAGACGGCGGCTATGAAACCTCAGATCCCGGTTATACAAACCGGAAGTGAGTACGGT
                  ***     *** *      ***** **  ***** * *************** * ***

RSG40475        TCTTTGGTTTATAAGGCTGGTGTGAGACAGGAAAGAGGTGTATTTCTTGATCCACACTCG
RSG50932        TCTCTGGTTTATAATCCCGGTTTTAGACAAGCAAGAGGTGGATTCCTTGATCCACACACG
                *** **********  * *** * ***** * ******** *** ************ **

RSG40475        ACTAAAATGGCTAGGATCAACAGGAAGAAGGCGATGATAAGATCAAGAAACAACTCTAGC
RSG50932        GCTAAGATGGCCAGGATCAACAGGAAGAAGGCGATGATAAGATCAAGAAACAACTCTAGC
                 **** ***** **************************************************

RSG40475        CCTAATTCTAGTTCGAATGAGTTGGTTGAGTCAAGGAGACAAGTGTCTCTTACCATGAAA
RSG50932        CCTAACTCTAGCACGAATGAGATGGTTGATTCAAGGAGACAAGTGGTTCTTAGCATGAAA
                ***** *****  ******** ******* ***************  ***** *******

RSG40475        ACCAATGCCGAGATTGCCGCTAGAAAAGATCTCTATCGATTCTCCTCATTCGATAACAAG
RSG50932        AATAGTGCCGAGATTGCCGCAAGGAAAGATCTCTATCGATTTTCCTCATTCGATAACAAG
                *  * *************** ** ****************** ******************

RSG40475        AAACTTAGGGTTTTGTTGGTGAAGCACTTGAAGAACAGTGATGTTGGGTCACTTGGGAGG
RSG50932        AAGCTTAGGGTTTTGCTGGTGAAACACTTGAAGAACAGCGATGTTGGATCACTTGGGAGG
                ** ************ ******* ************** ******** ************

RSG40475        ATCGTTCTACCAAAGAGAGAAGCAGAAGGAAATCTTCCGGAACTAACTGATAAAGAAGGA
RSG50932        ATCGTTCTACCAAAGAGAGAAGCTGAAAGAAATCTTCCGGAGCTATCTACTAAAGAAGGA
                ********************** *** ************* *** **  **********

RSG40475        ATGGTATTAGAGATGAAAGATGTTGACTCTGTGCAGTCTTGGGTACTGGTCCAATAACAA
RSG50932        ATGATAGTAGAGATGAGAGATGCTGACTCTATGCAGAATTG--TTCTGGTCCAATAACAA
                *** ** ********* ***** ******* *****  ***  * ***************

RSG40475        GAGCAGAATGTATGTCCTCGAAAACACAGGAGAATTTGTGAAGAAAAATGGAGTATTGAT
RSG50932        GAGCAGAATGTATGTCCTCGAAAACACAGGTAATTAA----------------------
                ******************************  * *
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**Appendix 5 GCMA Settings**

[AOC-20i+s] # of Rinses with Presolvent:2 # of Rinses with Solvent(post):4 # of Rinses with Sample:1 Plunger Speed(Suction):Middle Viscosity Comp. Time:0.2 sec Plunger Speed(Injection):High Syringe Insertion Speed: High Injection Mode: Normal Pumping Times:3 Inj. Port Dwell Time:0.0 sec Terminal Air Gap: No Plunger Washing Speed: High Washing Volume:8uL Syringe Suction Position:0.0 mm Syringe Injection Position:0.0 mm Solvent Selection: only A

[GC-2010] Column Oven Temp.:160.0 °C Injection Temp.:250.00 °C Injection Mode: Split Flow Control Mode: Linear Velocity Pressure: 105.8 kappa Total Flow: 59.1 mL/min Column Flow: 1.10 mL/min Linear Velocity: 40.0 cm/sec Purge Flow: 3.0 mL/min Split Ratio: 50.0 High Pressure Injection: OFF Carrier Gas Saver: ON Carrier Gas Saver Split Ratio: 10.0 Carrier Gas Saver Time: 1.00 min Splitter Hold: OFF Oven Temp. Program Rate Temperature (°C) Hold Time (min) - 160.02.00 4.00200.00.00 6.00224.02.00

< Ready Check Heat Unit > Column Oven: Yes SPL1: Yes MS: Yes < Ready Check Detector(FTD) > < Ready Check Baseline Drift > < Ready Check Injection Flow > SPL1 Carrier: Yes SPL1 Purge: Yes < Ready Check APC Flow > < Ready Check Detector APC Flow > External Wait: No Equilibrium Time:0.5 min

[GC Program]

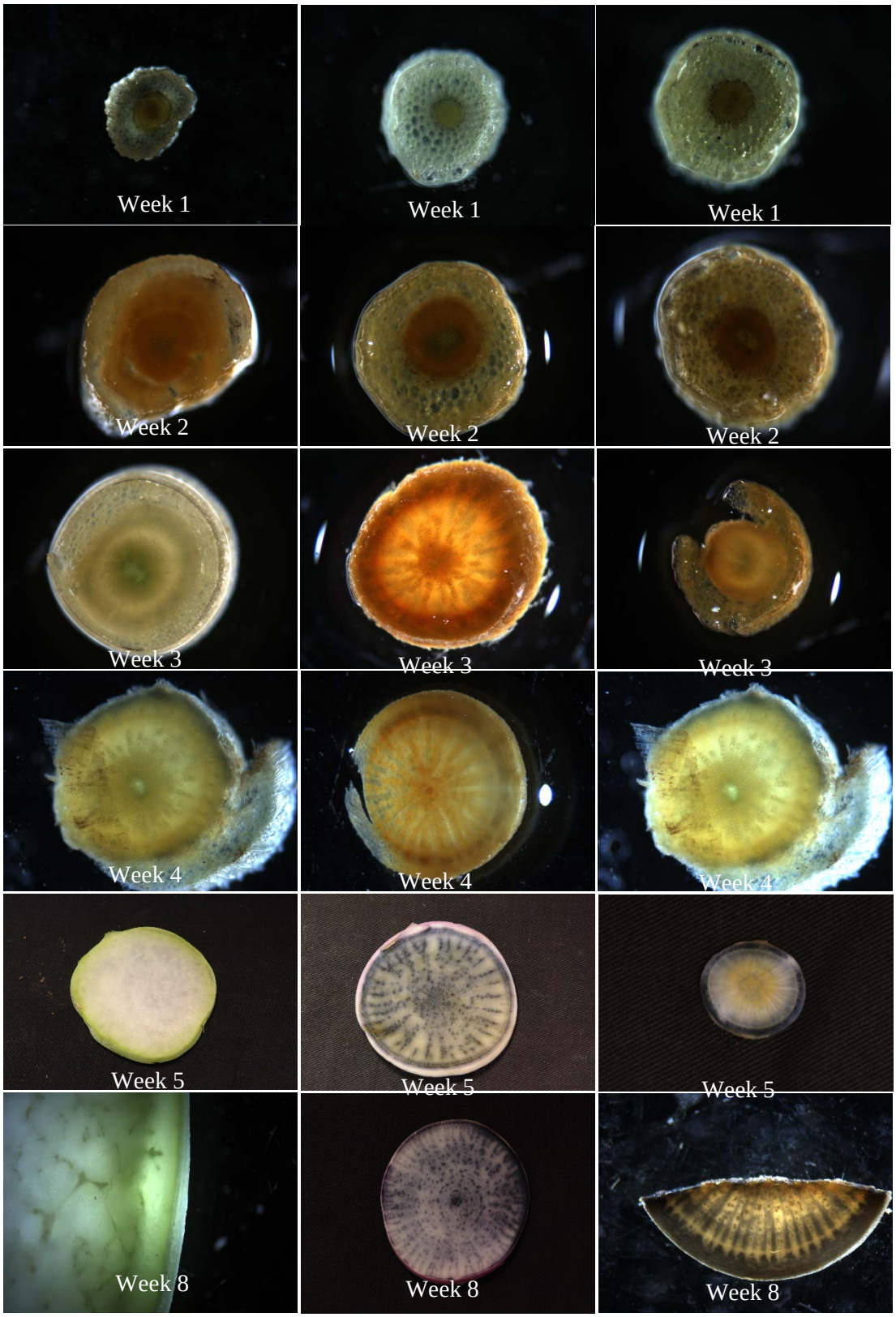
[GCMS-QP2010 Plus] IonSourceTemp:200.00 °C Interface Temp.:250.00 °C Solvent Cut Time:2.00 min Detector Gain Mode:Relative Detector Gain:0.00 kV Threshold:0

[MS Table] --Group 1 - Event 1--Start Time:2.20min End Time:18.00min ACQ Mode:Scan Event Time:0.20sec Scan Speed:2500 Start m/z:45.00 End m/z:500.00

Sample Inlet Unit:GC

[MS Program] Use MS Program:OFF

**Appendix 6 Additional sectional series – Starch**



**Appendix 7 Additional sectional series – Lipids**